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(54) Title: METHODS OF IDENTIFYING AND SCREENING GENES ASSOCIATED WITH INCREASED LONGEVITY AND SLOWED AGING

(57) Abstract

The present invention provides methods of identifying polynucleotides and polypeptides associated with life extension in a transgenic nematode system. The invention also provides screening methods using the polynucleotides and polypeptides thus identified, including *tkr–1*, a kinase receptor gene from *C. elegans*.

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METHODS OF IDENTIFYING AND SCREENING GENES ASSOCIATED WITH INCREASED LONGEVITY AND SLOWED AGING

CROSS-REFERENCE TO RELATED APPLICATIONS

This applications claims the benefit of U.S. Provisional Application No. 60/051,983, filed July 9, 1997, which is incorporated herein by reference.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

Research relating to this invention was supported in part by grants from the National Institutes of Health (NIH) (AG08322, AG08761 and AA00195). The government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to methods of identifying and using genes involved in the aging process. More particularly, the invention provides methods to identify polynucleotides and polypeptides associated with an increased lifespan and/or the slowing of aging in a nematode transgenic system, as well as screening methods using these polynucleotides and polypeptides.

BACKGROUND OF THE INVENTION

As the proportion of the older population dramatically increases, age-related phenomena, including diseases and physical manifestations of aging, will become an ever more pressing issue. In the United States, the percentage of elderly individuals (those over 65) is larger than ever: approximately 12.5% versus 8.1% in 1950 and 4.1% in 1900. By the time the last cohort of "baby-boomers" reaches 65 around 2030, the percentage of elderly will be over 20%. However, relatively little is known about the causes of age-related phenomena.

Apart from the ever-present desire to retain "youth", aging is presenting very real problems in terms of diseases and quality of life. Aging-related diseases include cancer,

stroke, heart disease, osteoporosis, and diseases of the central nervous systems (including Alzheimer's and Parkinson's disease).

One promising system in which to study the mechanisms of aging is the nematode *C. elegans*, a self-fertilizing hermaphroditic nematode species that is used in genetic research. The only mutants directly identified on the basis of extended longevity in any metazoan have been isolated in *C. elegans*. The self-fertilization minimizes inbreeding depression and allows rapid inbreeding, which facilitates the isolation of mutants and genetic analyses (Russell and Jacobson, 1985 In: *Handbook of the Biology of Aging* (2nd ed.), Finch, C.E. and Schneider, E.L. (Editors), pp. 128-145, Van Nostrand Reinhold, New York; Johnson, 1990 in: *Handbook of the Biology of Aging* (3rd ed.), Schneider, E.L. and Rowe, J.W. (Editors), pp. 45-59, Academic Press, New York; Johnson and Lithgow, 1992 *J. Am. Gerontol. Soc.* 40, 936-945; Lithgow, 1996 In: *Handbook of the Biology of Aging* (4th ed.), Schneider, E.L. and Rowe, J.W. (Editors), pp. 55-73, Academic Press, New York).

In the past, two approaches have been used with *C. elegans* to isolate genetic variants having longer life spans: (1) the production of recombinant-inbred (RI) strains, and (2) the isolation of mutants having extended life spans.

Recombinant Inbred Strains. Only slight progress in isolating long-lived worms has been made from the analysis of RI strains. Ebert et al., 1993 *Genetics* 135, 1003-1010, analyzed a large population of worms from crosses between the strains N2 and Bergerac that were segregating a variety of age-related genotypes. They carefully maintained polymorphisms and then examined multiple markers throughout the genomes of individual worms, comparing marker distribution in the longest-lived worms with the distribution of markers obtained from young worms. They found several regions of the genome that were associated with increases in lifespan.

Two studies (Brooks and Johnson, 1991 *Heredity* 67, 19-28; Shook et al., 1996 *Genetics* 142, 801-817) have used a series of RI strains derived from the same two parents to study aging. Intermediate levels of heritability (0.05 to 0.36) for lifespan and for fertility were found confirming earlier studies (Johnson and Wood, 1982 *Proc. Natl. Acad. Sci. USA* 79, 6603-6607; Johnson, 1987 *Proc. Natl. Acad. Sci. USA* 84, 3777-3781). Quantitative trait loci (QTL) were found for hermaphrodite self-fertility on linkage groups 2, 3, and 4 and QTLs for life span on groups 2 and 4 and the X chromosome. Little

evidence was found to suggest that reduced fertility was associated with increased life span, except for a locus on chromosome 2 where a major QTL affected both fertility and lifespan, both in the same direction.

Mutant strains. A second approach to studying the aging processes in *C. elegans* has been the isolation of long-lived mutants (Klass, 1983 *Mech. Aging Dev.* 22, 279-286). The first mutant identified (*age-1*) causes a 70% increase in life expectancy (Johnson, 1990, *Science* 249, 908-912), maps to the middle of chromosome 2, and has little affect on fertility, length of reproduction, or rate of development (Friedman and Johnson, 1988 *Genetics* 118, 75-86; Johnson and Lithgow, 1992, supra; Johnson et al.. 1993 *Genetica* 91, 65-77). The mutant allele slows the exponential rate of increase of the mortality rate (Johnson, 1990, supra). Mutant strains containing *age-1* move better at all ages and keep moving until later in life, suggesting that mutations that increase longevity increase overall vitality and "health" (Duhon and Johnson, 1995 *J. Gerontol. Biol. Sci.* 50, B254-B261).

There are other published reports of mutations in *C. elegans* that result in longer life. (1) Mutations in *spe-26* result in life extensions of about 80% for the hermaphrodite and the mated male (Van Voorhies, 1992 *Nature* 360, 456 458) presumably because of decreased sperm formation. (2) At 16°C and normal oxygen tension, *rad-8* mutant worms live almost 50% longer than wild-type worms (Ishii *et al.*, 1994, *J. Gerontol. Biol. Sc.* 49, B117-B120). Most or all of the increased life span is the result of an extended developmental phase. (3) *daf-2* mutants result in a more than twofold extension of mean life span at the permissive temperature (Kenyon et al., 1993 *Nature* 366, 461-464), and this extension is blocked by the action of the *daf-16* mutation. *daf-12* mutants interact with *daf-2* to cause an almost fourfold increase in mean life span (Larsen et al., 1995 *Genetics* 139, 1567-1583). These mutations affect the dauer formation pathway. The dauer is an alternative developmental path taken by *C. elegans* under "hard times" conditions (Riddle, 1988 In: *The Nematode* Caenorhabditis elegans, Wood, W.B. (Editor), pp. 393-412, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). (4) Larsen et al., 1995 *Genetics* 139, 1567-1583 showed that other dauer mutants also affect length of life: *daf-23* doubles the normal adult life span. (5) Mutations in *clk-1* and other *clk* genes alter development, the cell cycle and some alleles prolong life (Wong et al., 1995 *Genetics* 139, 1247-1259; Lakowski and Hekimi, 1996 *Science* 272, 1010-1013; Eubank *et al.*, 1997 *Science* 275, 980-983). These dauer-formation mutants define a signal transduction pathway in which

homologs to mammalian genes involved in signal-transduction can be identified. *daf-1* codes for a translational product that has homology to a serine/threonine kinase in the raf superfamily and which may be a cell-surface receptor (Georgi et al., 1990 *Cell* 61, 635-645). *daf-4* is the nematode homolog of human bone morphogenetic protein (BMP) receptor (Estevez et al., 1993 *Nature* 365, 644 649). *age-1/daf-23* has recently been shown to encode a P13 kinase (Morris et al., 1996, *Nature* 382, 536-539).

The relationship between *age-1* and the other age genes is not completely clear. *age-1 daf-16* double mutants have wild-type or shorter life spans similar to *daf-16* alone (Murakami and Johnson, 1996 *Genetics* 143, 1207-1218). The long-life trait of all other mutants described above is suppressed by *daf-16*, as are the stress resistance effects (see below). These gerontogenes are part of a genetic pathway that shares the *daf-16* gene downstream. Three gerontogenes have been cloned, daf-23 phosphatidylinositol-3-OH kinase (Morris et al., 1996, supra.), spe-26/actin associated protein (Varkey et al., 1995 *Genes & Develop.* 9, 1074-1086) and daf-2. Though controversial, *age-1* may be *daf-23*. It is probable that these gerontogenes negatively regulate stress resistance and life extension. Genes that have an opposite function (positive regulation of stress response and longevity) to these gerontogenes have not been isolated or characterized.

Involvement of Stress Response in Determining Life Span in C. elegans. Larsen (1992) *Proc. Natl. Acad. Sci. USA* 90, 8905-8909, and Vanfleteren (1993) *Biochem. J.* 292, 605-608 showed that *age-1* mutants are resistant to oxidative stress and have elevated levels of superoxide dismutase (SOD) and catalase in late life. Worms with the *age-1* mutation survive thermal stress better than do wild type worms (Lithgow et al., 1994 *J Gerontol. Biol. Sci.* 49 B270-B276; 1995 *Proc. Natl Acad. Sci. USA* 92, 7540-7544). This phenotype has been called increased thermotolerance (Itt). Itt was mapped to the *age-1* interval on chromosome 11 (Lithgow et al., 1995, supra) and was shown to be associated with *age-1* in both sterile and nonsterile worms (Lithgow et al., 1994, supra). New *age-1* mutants are all Itt, as well as UV-resistant and resistant to reactive oxidants (Duhon et al., 1996 *Dev. Genet.* 18, 144-153). Other Age strains, Including constitutive dauer mutations, *daf-2*, *daf-28*, *daf-4*, and *daf-7* are also Itt (Lithgow et al., 1995, supra). Very recent studies have also shown that all long-lived mutants in *C. elegans* are UV resistant as well (Murakami and Johnson, 1996, supra).

Life-extension mutants must have altered genes specifying the rate-determining process(es) for life span. The observation that repair processes are increased to higher levels in many longevity mutants suggests that these are the rate-determining processes limiting longevity in a variety of species.

A "gerontogene" is a gene that can be altered to slow aging, extend life and enhance late-life health. Genes affecting aging have also been called "longevity assurance genes" (D'mello et al., 1994 *J. Biol. Chem.* 269, 15451-15459) or "longevity associated genes" by the NIA (McCormick, personal communication) both of which can be conveniently abbreviated using the term "LAG." This term is similar to the term "gerontogene" as used by Rattan (1985) *Bioessays 2*, 226-228.

These classes of genes are a starting scheme for classifying genes involved in aging and senescence. Null mutants in any of the first three types of genes should result in life extension while a null mutant in the last class should result in life shortening. Notably, all life-extension mutants previously identified in *C. elegans* result from hypo- or nullomorphic mutations.

The longevity genes, or life-extension genes, are of obvious interest and importance, both for their life-extension potential and the possibility of their contributing to the enhancement of the quality of life, particularly later during the lifespan. However, very few of these genes have been identified, and even less is understood about how these genes act to prevent aging and promote life extension.

There is an increasingly pressing need to discover genes whose function is associated with life-extension and/or stress resistance, particularly those genes which positively regulate life extension (i.e., genes in which over-expression confers an increased life span). Such genes and their products could provide the basis for, inter alia, screening for promising anti-aging (including anti-disease) agents.

Throughout this application, various publications, patents, and published patent applications are referred to by an identifying citation. The disclosures of these publications, patents, and published patent specifications referenced in this application are hereby incorporated by reference in their entirety into the present disclosure.

SUMMARY OF THE INVENTION

This invention is based upon the identification of genes (i.e., polynucleotide sequences and the polypeptide sequences encoded therein) which are associated with life extension, particularly those genes which positively modulate life extension. Application of these methods has identified a gene from *C. elegans*, designated *tkr-1*, which is associated with life-extension as well as stress resistance.

Accordingly, the present invention provides methods of identifying a polynucleotide associated with longevity comprising measuring lifespan in a transgenic nematode, such as *C. elegans*, said transgenic nematode comprising the polynucleotide, wherein a polynucleotide is identified as associated with longevity if its expression confers longer lifespan than a suitable control. In some embodiments, the method comprises 1) computer similarity searches for known gerontogenes or genes suspected to be involved in stress resistance or aging, and 2) determining the life-span increasing capability (if any) of the genes in transgenic nematodes. Further provided is a method for identifying genes leading to increased stress resistance in an organism, in particular, heat shock, UV resistance and oxidative resistance. Such genes may increase lifespan in organisms containing the gene or provided with the product encoded thereby.

In another aspect of the invention, the life-extension gene (i.e., polynucleotide) and/or the protein encoded thereby could be used to discover drug candidates that are targeted to the gene, or to the protein encoded thereby, as site-specific interventional agents. Accordingly, the invention provides screening methods in which an agent is identified by its ability to modulate the life-extension polynucleotide and/or polypeptide function(s) (i.e., functional characteristic). Accordingly, the present invention encompasses methods of identifying agents that may have life-extension activity comprising contacting a transfected cell comprising a life-extension polynucleotide identified in the methods described herein or a polypeptide encoded by the life extension polynucleotide with the agent, wherein the agent is identified by its ability to modulate a functional characteristic of a life-extension polynucleotide or polypeptide.

In a further aspect of the invention, a transgenic nematode is provided, wherein the transgene is a chimeric *tkr-1* gene. Such chimeric *tkr-1* transgenes may comprise, for example, sequences encoding a human FGF receptor kinase domain. Also provided is a transgenic nematode comprising a *tkr-1* polynucleotide. A transgenic nematode of the

present invention can be used in the screening methods described herein. The present invention also provides a chimeric nematode *tkr-1* gene.

Other objects of the present invention will be readily apparent to those of ordinary skill in the appropriate art.

DESCRIPTION OF THE FIGURES

Fig. 1 is a schematic depicting the physical map positions cosmids and YACs initially tested in the transgenic system. Transgenic *C. elegans* were constructed carrying multiple-copies of the YAC (thick bar) or cosmid DNA (thin bar) in order to over-produce the sequences of interest. The bar indicates DNA clones that confer (black) and do not confer (white) increased resistance to heat. The lower section is an amplified region showing the organization of *tkr-1*. The abbreviations of the restriction sites are as follows: Bg (*Bgl II*), Bs (*Bstx I*), E (*Eco RI*), H (*Hind III*) and Sp (*Spe I*).

Fig. 2 is a schematic as in Fig. 1, with additional data regarding a frameshift and a deletion *tkr1* constructs. Parentheses show numbers of independent transgenics per total that display increased resistance to heat, UV and life extension. The frameshift mutant gene, *tkr-1*Δkin, lacks the cytoplasmic domain carrying the kinase region of the protein encoded thereby (see Fig. 4 for detail). The truncation mutation, *tkr-1ΔBB*, lacks the whole coding region except for the N-terminal 15 amino acids, as shown schematically in Figure 3.

Fig. 3 is a schematic summarizing all *tkr-1* derivative constructs as well as the lifespan and stress (UV and thermotolerance) data. Designations are as in previous figures. *tkr-1FGFR* is a construct in which the kinase domain from fibroblast growth factor receptor has been substituted for the kinase domain of *tkr-1*. The percentage shown represent the increase in mean life span (Life), UV resistance (UV), or thermotolerance (Heat). Abbreviations: TM, transmembrane domain; TK1, kinase domain 1; TK2, kinase domain 2, KI, kinase insert.

Fig. 4 shows the nucleotide (SEQ ID NO:12) and predicted amino acid sequence (SEQ ID NOS:13-23) of *tkr-1*. The predicted transmembrane domain is underlined. Four bp of a frameshift mutation was introduced in the *Bgl II* site resulting in the deletion of a kinase region (tkr-1Δkin; arrows). The potential autophosphorylation sites are circled.

Figs. 5(a) - (c) show the alignment of the amino acid sequences (SEQ ID NOS:24-81) of the kinase domain of *tkr-1* and *tkr-2*. The *tkr-1* nematode gene family members and related mammalian receptor kinases are shown. Identical residues are shown by filled-in boxes with white letters.

Fig. 6 is a sequence comparison of *tkr-1* and *tkr-2* with human fibroblast growth receptor 1 (FGFR-1) (SEQ ID NOS:82-99). The positions of the predicted signal peptide (amino acids 18-29) and the HRDLALRN motif (amino acids 319-326) specific to the kinase subdomain VIB are shown by a line above and a dotted line below, respectively. A potential cleavage site of the signal peptide is shown by the arrowhead. Predicted autophosphorylation sites are shown by asterisks. Abbreviations: TM, transmembrane domain; TK1, kinase domain 1; TK2, kinase domain 2; KI, kinase insert.

Fig. 7 is a schematic depicting the evolutionary history of kinase domains most closely related to the kinase domain of *tkr-1*.

Figs. 8 (a) and (b) shows increased survival of *tkr1-1* and *tkr1-2* as compared to controls and to *tkr-2* (*tkr2-1* and *tkr2-2*). Fig. 8(a) shows increased survival after heat shock; Fig. 8(b) shows increased survival after UV exposure.

Figs. 9 (a) and (b) are graphs depicting life extension conferred by the *tkr-1* gene *(tkr1-1* and *tkr1-2*; Fig. 9 (a)) when compared to controls and to *tkr-2* (*tkr2-1* and *tkr2-2*(Fig. 9 (b)).

Figs. 10 (a) and (b) are schematic depictions of tandemly clustered *tkr-1* family members.

Figs. 11 (a) – (c) are graphs depicting increased thermotolerance, UV resistance and life span of animals expressing the construct encoding the hybrid *tkr-1*-FGFR, containing the kinase domain from human FGF-R1. Panel a shows that thermotolerance is increased by *tkr1-FGFR* ($p<0.0001$). Panel b shows that UV resistance is increased by *tkr1-FGFR* ($p<0.0001$). Panel c shows that life is extended by the chimeric construct, *tkr-FGFR* ($p<0.0001$). Each panel also depicts the lack of effect of animals carrying a deletion of the kinase domain (*tkr-1Δkin)*.

Figure 12 is a graph depicting suppression of the effects of *tkr-1* by a reduction of function mutation of *daf-16*. The *daf-16(m26)* mutation is epistatic to the *tkr-1* phenotypes.

Fig. 13 is a schematic depicting a construct encoding a *tkr-1*-green fluorescent protein (GFP) fusion protein.

Fig. 14 (a) and (b) are graphs depicting the response of transgenic animals carrying the construct encoding a *tkr-1*-green fluorescent protein (GFP) fusion protein, shown schematically in Figure 13, after exposure to UV (upper panel) or heat (lower panel). Induction of *tkr-1* was measured as the intensity of GFP fluorescence. Starvation also induces expression of the *tkr-1*-GFP fusion protein in these animals.

Figure 15 shows an amino acid sequence (SEQ ID NO:100) of the kinase domain of human FGFR.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered an effective method to identify polynucleotides associated with life extension, particularly those polynucleotides which positively modulate life extension (i.e., overexpression positively modulates life extension). This powerful transgenic nematode system can provide polynucleotides which in turn can be used in screening methods to identify agents which may be useful in treating aging-related phenomena and/or delaying the aging process and/or lengthening life span. One such polynucleotide, denoted *tkr-1*, has been identified. Overexpression of *tkr-1*, which encodes a putative receptor tyrosine kinase, increases longevity 40-100% (average, 65%) and confers increased resistance to heat and ultraviolet light in transgenic nematodes, and does not alter development or fertility. Unlike previously identified gerontogenes, *tkr-1* positively modulates longevity and stress resistance. The *tkr-1* polynucleotides described herein share a region of homology with a part of a human gene, FGFR (fibroblast growth factor receptor), and the FGFR kinase domain functionally substitutes for the kinase domain of *tkr-1*. Polynucleotides and polypeptides identified by these methods may likely function to modulate a variety of other target genes to slow aging. Thus, identification of these polynucleotides and the polypeptides encoded thereby is useful for locating other such similar genes, including those in other organisms such as mammals and including humans.

In another aspect of the invention, the polynucleotides identified as associated with life extension (and/or polypeptides encoded thereby) can be used in screening assays to discover drugs that modify the activity of such polynucleotides, or the polypeptides

encoded by these polynucleotides, to produce site-specific interventional agents. In some embodiments, the transgenic nematode may be used for screening, especially the transgenic nematode comprising a *tkr-1* polynucleotide or the kinase domain of *FGFR*..

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. *See, e.g.*, Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, (F.M. Ausubel et al. eds., 1987); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (M.J. McPherson, B.D. Hames and G.R. Taylor eds., 1995); ANIMAL CELL CULTURE (R.I. Freshney. Ed., 1987) and METHODS IN CELL BIOLOGY, VOL. 48, CAENORHABDITIS ELEGANS: MODERN BIOLOGICAL ANALYSIS OF AN ORGANISM, (H.F. Epstein and D.C. Shakes eds., 1995, Academic Press, San Diego).

Definitions

A polynucleotide or polypeptide that is "associated with longevity" is one whose function correlates with increased lifespan. "Longevity" and "life-extension", used interchangeably herein, also include delay and/or stabilizing the aging process. Preferably, the longevity is due to an extension of the mature life phase, as opposed to an extension of the immature life phase (i.e., delay in maturity). Such polynucleotides or polypeptides that are found to be correlated with longevity are generically denoted herein as "life-extension" polynucleotides or polypeptides.. A "function" of a polynucleotide can be on any level, including DNA binding, transcription, translation, processing and/or secretion of expression product, interaction (such as binding) of expression product with another moiety, and regulation (whether repression or de-repression) of other genes. It is understood that a life-extension polynucleotide or polypeptide includes fragments, or

regions, of a polynucleotide or polypeptide, as long as the requisite life-extension phenotype is observed.

The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes single-, double-stranded and triple helical molecules. "Oligonucleotide" refers to polynucleotides of between about 5 and about 100 nucleotides of single- or double-stranded DNA. Oligonucleotides are also known as oligomers or oligos and may be isolated from genes, or chemically synthesized by methods known in the art.

The following are non-limiting embodiments of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid molecule may also comprise modified nucleic acid molecules, such as methylated nucleic acid molecules and nucleic acid molecule analogs. Analogs of purines and pyrimidines are known in the art, and include, but are not limited to, aziridinycytosine, 4-acetylcytosine, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, pseudouracil, 5-pentylnyluracil and 2,6-diaminopurine. The use of uracil as a substitute for thymine in a deoxyribonucleic acid is also considered an analogous form of pyrimidine.

If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates,

phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s).

Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH groups can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups.

Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, but not limited to, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside.

Although conventional sugars and bases will be used in applying the method of the invention, substitution of analogous forms of sugars, purines and pyrimidines can be advantageous in designing a final product, as can alternative backbone structures like a polyamide backbone.

As used herein, the term "*tkr-1* gene(s)" or "*tkr-1*" refers to the *C. elegans tkr-1* gene. As is understood in the art, the *tkr-1* gene includes, not only the coding sequences, but also 5' and 3' flanking sequences, as shown in SEQ ID NO: 1. A "fragment" of *tkr-1* is a portion of the *tkr-1* gene, and as such may contain coding and/or non-coding sequences. Preferably, a fragment of *tkr-1* comprises at least 10 contiguous nucleotides, more preferably at least 15, more preferably at least 25, more preferably at least 30, more preferably at least 50, more preferably at least 100 contiguous nucleotides.

"tkr-1" refers to a protein (polypeptide) product encoded in the *C. elegans tkr-1* gene. The sequence of full-length tkr-1 is shown in SEQ ID NO: 2 as well as in Figure 4. A "fragment" of full-length tkr-1 is a portion of the *tkr-1* gene product.

A *"tkr-1* polynucleotide" refers to any of the polynucleotide embodiments described herein based on the *tkr-1* gene polynucleotide sequence (SEQ ID NO:2). A *"tkr-1"* polynucletoide also includes fusion or hybrid constructs, such as those described herein. A "tkr-1 polypeptide" refers to a polypeptide product encoded by or within *tkr-1;* thus, a"tkr-1 polypeptide" refers to any of the polypeptide embodiments described herein based on the coding region of *tkr-1*, including full-length tkr-1 and fragments of tkr-1.

"Isolated" polynucleotides or polypeptides are substantially free of those substances with which they are associated in nature. "Substantially free" refers to compositions containing less than about 50%, preferably less than about 70%, and more preferably less than about 90% of the materials with which they are usually associated with in nature.

The term "vector" refers to a DNA molecule that can carry inserted DNA and be perpetuated in a host cell. Vectors are also known as cloning vectors, cloning vehicles or vehicles. The term includes vectors that function primarily for insertion of a nucleic acid molecule into a cell, replication vectors that function primarily for the replication of nucleic acid, and expression vectors that function for transcription and/or translation of the DNA or RNA. Also included are vectors that provide more than one of the above functions.

A "host cell" includes an individual cell, cell culture or cell which is part of a whole animal which can be or has been a recipient for vector(s) or for incorporation of nucleic acid molecules and/or proteins. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent due to natural, accidental, or deliberate mutation. A host cell includes cells transfected with the polynucleotides of the present invention.

The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acids and does not refer to a specified length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within this definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids), polypeptides with substituted linkages, as

well as other modifications known in the art, both naturally and non-naturally occurring. "Analogs" of tkr-1 proteins are any naturally or non-naturally occurring peptide or peptide mimicking molecule which exhibit the same biological effects as tkr-1 proteins, for example increasing life span.

A "individual " is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. The term includes transcription as well as translation.

A "transgenic" animal refers to a genetically engineered animal or offspring of genetically engineered (i.e., altered) animals. As used herein, a transgenic nematode may or may not be genetically altered by using a polynucleotide from at least one unrelated organism, such as from a virus, plant, or other animal. Thus, a "transgenic nematode" is one that has been stably transfected with heterologous (foreign) or native (self) polynucleotide. A "transgene" is any gene that is inserted to create a transgenic animal. The transgene can be homologous or heterologous. For example, a homologous transgene is shown *infra*, namely a stress-related gene such as *tkr-1*. A heterologous transgene can be, for example, a chimeric gene which is created by insertion of a heterologous sequence that does not normally occur in the host animal, *e.g.*, nematode. An example of a heterologous transgene is the *tkr-1*FGF-R1 construct described herein.

"Transformation" or "transfection" refers to the insertion of an exogenous polynucleotide into a host cell or animal, irrespective of the method used for the insertion, for example, lipofection, transduction, infection or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome.

As used herein, "expression" includes transcription and/or translation.

As used herein, the term "agent" means a biological or chemical compound such as a simple or complex organic or inorganic molecule, a peptide, a protein, oligonucleotide, polynucleotide, carbohydrate, or lipoprotein. A vast array of compounds can be synthesized, for example oligomers, such as oligopeptides and oligonucleotides, and synthetic organic compounds based on various core structures, and these are also included

in the term "agent". In addition, various natural sources can provide compounds for screening, such as plant or animal extracts, and the like. Compounds can be tested singly or in combination with one another.

An agent that "modulates" life-extension is an agent that affects life-extension, or lifespan, whether directly or indirectly, whether negatively or positively.

Methods to identify polynucleotides associated with longevity

The present invention provides methods of identifying a polynucleotide associated with longevity comprising measuring lifespan in a transgenic nematode, said transgenic nematode comprising the polynucleotide, wherein the polynucleotide is identified as associated with longevity if its expression confers longer lifespan than a suitable control. The method used to identify gerontogenes whose over-expression causes increased stress resistance and increased life span could be effective for isolating such genes in mammals and other organisms. Genomic screening of transgenic animals would also be useful to identify sequences known or suspected to be associated with the stress response which are also associated with longevity.

The methods of this invention identify particularly useful sequences in that their over-expression is associated with longevity, i.e., their over-expression positively modulates life extension. Polynucleotides are overexpressed in the transgenic nematode, leading to increased lifespan. Accordingly, transgenic worms carrying cosmid or YAC clones as set forth in the Figures were constructed, and tested whether the transgenic worms showed life extension as well as an increased stress resistance in young adult hermaphrodites.. As detailed in the Examples below, the genomic screening specifically covered a 0.5 Mb region to the left of unc-4 on chromosome II of the nematode *Caenorhabditis elegans*. A gene, *tkr-1*, with a newly defined role in stress response and longevity was identified. The nucleotide sequence of tkr-1 and also the amino acid sequence of the polypeptide encoded thereby is shown in Figure 4.

The methods entail measuring the lifespan of a transgenic nematode, preferably *C. elegans*, that contains the polynucleotide to be tested. It is understood that, for purposes of this invention, a polynucleotide identified by conferring increased lifespan (when compared to a suitable control) means that any polypeptide encoded by that polynucleotide has also been identified. It is also understood that fragments, or regions, of the

polynucleotides and/or polypeptides encoded therein may also be identified by testing for lifespan conferring function as described herein. Such polynucleotides and polypeptides identified by these methods are denoted "life-extension polynucleotide(s)" and "life-extension polypeptide(s)" herein.

The polynucleotide(s) to be screened may be from any source, and of any function, either known or unknown. The polynucleotide may or may not be derived from a nematode such as *C. elegans* and may be, for example, mammalian, preferably human. The significant degree of homology (i.e., shared sequences) across species and even various genuses indicates that this screening method would be suitable for using polynucleotide sequence from sources other than nematodes such as *C. elegans*. Similarly, *C. elegans* sequences may indicate other mammalian sequences that could be tested for life-extension properties. We have shown, for example, that the kinase domain from FGFR can substitute for the kinase domain from *tkr-1* in terms of life extension and stress resistance.

A polynucleotide to be tested may be prepared by any technique known to those of skill in the art using the polynucleotide sequences provided herein. For example, they can be prepared by isolating the polynucleotides from a natural source, or by chemical synthesis, or by synthesis using recombinant DNA techniques.

Methods of making transgenic nematodes, such as *C. elegans*, are described in the Examples. These methods are also applicable to other nematodes, for example *C. briggsae*. Generally, any acceptable method of introducing the polynucleotide of interest so that it is stably inherited in the organism is acceptable. The polynucleotide is preferably introduced by direct injection into the gonads of the nematode. Generally, a selectable marker, such as the *rol-6 (su1006)* gene, which confers the rolling phenotype is co-transfected to detect transfection. Stable transgenics are then selected and propagated for testing.

The polynucleotide is introduced to the nematode in any one of a number suitable vectors, such as cloning and/or expression plasmids, BACs, YACs, cosmids, many of which are commercially available (Examples 1 and 4). Such vectors are known in the art and need not be described in detail herein.

The ability of the polynucleotide to confer increased lifespan is measured and compared to a suitable control. For purposes of the present invention, a suitable control is

one which does not over-express the polynucleotide. "Over-expression" is a term well-understood in the art, and, in this context of transgenic *C. elegans*, means that there is a copy number of the polynucleotide higher than that generally found in nature. "Over-expression" includes transcription and/or translation. Copy number can be readily measured (if necessary) by techniques such as Southern blotting. Degree of transcription can be measured using, for example, Northern analysis. Amount of expression product may be measured by Western analysis (if an antibody is available) or by a functional assay that detects the amount of protein, such as kinase activity. The polynucleotide's function(s) which is associated with life extension may be a coding and/or non-coding sequence (such as a transcriptional regulatory element). Generally, one skilled in the art would assume that a transgenic nematode, since it de facto contains an additional copy (or copies) of the polynucleotide, would "over-express" the polynucleotide. Methods of varying the number of copies are known in the art and include, for example, varying concentration during injection. Suitable controls include, but are not limited to, a transgenic nematode that contains a selectable marker only (i.e., has not been transformed with the polynucleotide to be tested) and a nematode that has not been transformed.

In some embodiments, polynucleotides known or suspected to be associated with a stress response are tested. In other embodiments, polynucleotides known or suspected to be associated with stress resistance are tested. Polynucleotides known or suspected to confer stress resistance may be obtained in a variety of ways, such as using those published in the literature or searching databases for sequences homologous to sequences known to be associated with stress resistance. As discussed below, a *C. elegans* polynucleotide (gene) *tkr-1* was identified using this method.

In other embodiments, *C. elegans* genes similar to the mammalian tyrosine receptor kinases that are involved in cellular stress response are used. Other stress response genes could also be used as a basis for the homolog screening. These include, for example, members of the MAP kinase family or other kinases, for example Jun N-terminal kinase (JNK/SAPK) in the cosmid T07A9, p38/HOG-1 in the cosmid B0218, ELK1 in the cosmid F19F10, CL100 in the cosmid C05B10, and transcription factors, for example, AP-1 (c-Fos/c-Jun) in the cosmid T24H10 and TCF/Elk-1. Several receptor kinases also share in (and perhaps cross-regulate) response to UV or other stimuli. Also included are mammalian homologs of these genes. These kinases include the c-kit protooncogene,

PDGFR (platelet derived growth factor receptors), IGF-R (insulin like growth factor I receptors), and EGF-R (epidermal growth factor receptors). For example, binding of c-kit to its ligand, stem cell factor (SCF), confers an increased radiation resistance to bone marrow progenitor cells (Shul et al., 1995 *Cancer Res.* 55, 3431-3437). Several additional receptor kinases also are activated or phosphorylated after exposure to UV irradiation and/or oxidative stress, including EGF-R. insulin receptor and T cell receptor (Schieven et al., 1994 *J. Biol. Chem.* 269. 20718-20721: Coffer et al., 1995 *Oncogene* 11, 561-569). These polynucleotide and polypeptide sequences are readily available in the literature and publicly accessible databases and need not be described herein.

Accordingly, the invention includes a method of identifying a polynucleotide associated with longevity comprising (a) conducting a database search using a polynucleotide (or polypeptide) sequence known to be (or suspected to be) associated with stress resistance; and (b) testing a polynucleotide identified in step (a) for ability to confer increased lifespan in a transgenic nematode comprising the polynucleotide.

If desired, additional tests may be conducted using the polynucleotide(s) identified above to further characterize the nature of the polynucleotide's function with respect to longevity. For example, egg laying may also be measured to determine whether the longevity occurs by delaying maturity. As another example, other phenotypes associated with other gerontogenes could be tested to determine whether the identified polynucleotide shares functional pathways with these other genes.

The invention also includes transgenic nematodes comprising (i.e., transfected with) any of the polynucleotide(s) identified herein.

tkr-1, a gene associated with longevity

Using the above methods, a gene denoted *tkr-1* (SEQ ID NO:12) was identified from overlapping cosmids showing life extension and stress resistance when introduced in high copy in *C. elegans*. This tyrosine kinase receptor gene, with sequence similarity to the mammalian c-kit protooncogene and fibroblast growth factor receptor gene, confers increased resistance to heat and ultraviolet light, and also increases longevity up to 65%. The cytoplasmic kinase domain of tkr-1 must be present to cause these forms of stress resistance. A tkr-1 homolog, tkr-2, promotes no detectable stress resistance and moderate life extension. Unlike the *C. elegans* gerontogenes previously identified (such as age-1,

daf-2, spe-26 and clk-1), *tkr-1* has an "anti-aging" function that positively modulates stress resistance and longevity.

The *tkr-1* gene was identified using the following two-part strategy: (1) performing a computer homolog search using the *C. elegans* database and (2) a genomic screening using transgenics, to identify an "anti-aging" gene that confers stress resistance and life extension. Genes conferring stress resistance in adult *C. elegans* when over-expressed were screened and then tested to see whether these genes extended the life span. The present inventors have also shown that the life extension and stress resistance effects of tkr-1 are suppressed by daf-16 mutants (Figure 12). In particular, the daf-16(m26) mutation is epistatic to the *tkr-1*-associated phenotypes. Without being bound by one theory, this suggests that *tkr-1* functions in the same genetic pathway utilized by other gerontogenes.

Because of their association with life extension, *tkr-1* polynucleotides and polypeptides are useful in screening agents which modulate tkr-1 function, and thus may be involved in the aging process, particularly in life extension. These uses are discussed below. Further, because of the significant discovery that the kinase domain of FGFR, particularly human FGF-R1, substitutes for the kinase domain of *tkr-1*, this implies that (a) the chimeric gene construct comprising *tkr-1* with the substituted FGFR kinase domain can be used in screening for potentially useful anti-aging agents; (b) the kinase domain of FGFR may have important functional implications in longevity, and thus may be a basis for screening for anti-aging agents; (c) FGFR may have important function in longevity, and thus polynucleotides encoding FGFR can be screened for anti-aging agents. These and other screening embodimennts are discussed below.

Screening methods using polynucleotides and/or polypeptides associated with longevity

The invention also provides methods of *in vitro* and *in vivo* screening using the life-extension polynucleotides identified above and/or any polypeptides encoded therein. The life-extension polypeptides and polypeptides to be used in these screening methods may be obtained using standard synthetic methods known in the art, including, but not limited to, isolation from natural sources, recombinant methods, chemical synthetic methods, and enzymatic digestion followed by purification. The screening methods generally employ an

expression construct comprising the polynucleotide(s), although in in vitro methods the polynucleotide(s) and/or polypeptide(s) alone may be used.

The correlation of over-expression of *tkr-1* with life extension indicates that the up-regulation of a functional characteristic of life-extension polynucleotide (discussed below) confers longevity, and that agents which likewise cause this up-regulation may be useful in treating age-related phenomena. Conversely, an agent which causes down-regulation is indicated as a toxic agent in the sense that it negatively affects longevity. Thus, the screening methods described herein are applicable to a number of contexts, including screening for potentially useful anti-aging substances as well as screening for potentially harmful age-inducing substances.

Further, our finding that the kinase domain of FGFR can substitute for the kinase domain of *tkr-1* implies shared functionality between the kinase domain of FGFR, and possibly FGFR, and *tkr-1* with respect to longevity and/or stress resistance. The conservation between *trk-1* and human receptor kinases indicates that they may share a downstream signaling pathway for increased longevity and/or stress resistance.

Accordingly, the present invention encompasses methods of identifying agents that may have life-extension activity based on their ability to modulate a functional characteristic of a life-extension polynucleotide and/or polypeptide. These methods may be practiced in a variety of embodiments. We have observed that polynucleotide sequences may be identified based on their ability to confer life extension when overexpressed in nematodes, particularly *C. elegans*. While not being bound to any one theory, this observation suggests that a pathway(s) involving these polynucleotide(s) (such as *tkr-1*, discussed below) may play a role in life extension. This further suggests that modulation of function of these life-extension polynucleotide(s) (including, but not limited to, modulation of any polypeptide(s) encoded therein) may result in control of the aging and/or survival process. Thus, an agent identified by the methods of the present invention may be useful in the treatment of aging-associated conditions. It is understood that these screening embodiments include, but are not limited to, using the *tkr-1* polynucleotides and polypeptides described above.

The methods described herein are *in vitro* and *in vivo* cell- and animal (*e.g.*, nematode)-based screening assays. In the *in vitro* embodiments, an agent is tested for its ability to modulate function of a life-extension polynucleotide and/or life-extension

polypeptide using the methods described herein. In the cell-based embodiments, living cells comprising the life-extension polynucleotide(s) and/or life-extension polypeptide(s) are used for testing agents. For purposes of this invention, an agent may be identified on the basis of modulation of a polynucleotide or polypeptide, although, given that such polynucleotides and/or polypeptides have been identified by their ability to confer life extension due to over-expression, will likely be preferably that the agents confer increased, or enhanced, functionality. These assays can also be conducted on whole animals, using for example, nematodes.

In all of these methods, modulation of function of a life-extension polynucleotide and/or polypeptide may occur at any level. An agent may modulate function by reducing or preventing transcription of a life-extension polynucleotide. An example of such an agent is one that binds to the upstream controlling region, including a polynucleotide sequence or polypeptide. An agent may modulate translation of mRNA. An example of such an agent is one that binds to the mRNA, such as an anti-sense polynucleotide, or an agent which selectively degrades or stabilizes the mRNA. An agent may modulate function by binding to the life-extension polypeptide. An example of such an agent is a polypeptide or a chelator. An agent may modulate function by affecting gene expression of a gene that is regulated by a life-extension gene. An example of such an agent is one that alters expression of a life-extension-regulated gene on any of the levels discussed above.

With respect to these screening methods, any life-extension polynucleotide (and or polypeptide) may be used. Generally, the life-extension polynucleotide (and/or polypeptide) will have been identified using the transgenic methods described above. In some embodiments, a *tkr-1* polynucleotide (and/or polypeptide) is used. In other embodiments, a fusion construct in which a polynucleotide encoding the kinase domain of FGFR has replaced the coding region for the kinase domain of *tkr-1* (such as that described in Example 4) is used. In other embodiments, a polynucletoide the kinase domain of FGFR is used. In other embodiments, a polynucleotide encoding FGFR is used. In other embodiments, mammalian, preferably human, homologs of the polypeptides identified above can be used. For clearness, these embodiments will generally not be reiterated below, as the various types of assays are discussed.

In vitro screening methods

In *in vitro* screening assays of this invention, an agent is screened in an *in vitro* system, which may be any of the following: (1) an assay that determines whether an agent is modulating transcription of a life-extension polynucleotide; (2) an assay for an agent which modulates translation of mRNA or a polynucleotide encoding a life-extension polypeptide; (3) an assay for an agent that binds to a life-extension polynucleotide or polypeptide.

For an assay that determines whether an agent modulates transcription of a life-extension polynucleotide, an in vitro transcription or transcription/translation system may be used. These systems are available commercially, and generally contain a coding sequence as a positive, preferably internal, control. A life-extension polynucleotide is introduced and transcription is allowed to occur. Comparison of transcription products between an in vitro expression system that does not contain any agent (negative control) with an *in vitro* expression system that does contain agent indicates whether an agent is affecting transcription. Comparison of transcription products between control and the life-extension polynucleotide indicates whether the agent, if acting on this level, is selectively affecting transcription of the life-extension polynucleotide (as opposed to affecting transcription in a general, non-selective or specific fashion).

For an assay that determines whether an agent modulates translation of a life-extension mRNA or a polynucleotide encoding a life-extension polypeptide, an *in vitro* transcription/translation assay as described above may be used, except the translation products are compared. Comparison of translation products between an *in vitro* expression system that does not contain any agent (negative control) with an *in vitro* expression system that does contain agent indicates whether an agent is affecting transcription. Comparison of translation products between control and the life-extension polynucleotide indicates whether the agent, if acting on this level, is selectively affecting translation of the life-extension polynucleotide (as opposed to affecting translation in a general, non-selective or specific fashion).

For an assay for an agent that binds to a life-extension polypeptide, a life-extension polynucleotide is first recombinantly expressed in a prokaryotic or eukaryotic expression system as a native or as a fusion protein in which a life-extension polypeptide (or fragment thereof) is conjugated with a well-characterized epitope or protein as are well known in the

art. Recombinant life-extension polypeptide is then purified by, for instance, immunoprecipitation using anti-life-extension polypeptide antibodies or anti-epitope antibodies or by binding to immobilized ligand of the conjugate. An affinity column made of life-extension polypeptide or life-extension polypeptide fusion protein is then used to screen a mixture of compounds which have been appropriately labeled. Suitable labels include, but are not limited to flurochromes, radioisotopes, enzymes and chemiluminescent compounds. The unbound and bound compounds can be separated by washes using various conditions (e.g. high salt, detergent) that are routinely employed by those skilled in the art. Non-specific binding to the affinity column can be minimized by pre-clearing the compound mixture using an affinity column containing merely the conjugate or the epitope. A similar method can be used for screening for agents that competes for binding to a life-extension polypeptide. In addition to affinity chromatography, there are other techniques such as measuring the change of melting temperature or the fluorescence anisotropy of a protein which will change upon binding another molecule. For example, a BIAcore assay using a sensor chip (supplied by Pharmacia Biosensor, Stitt et al. (1995) *Cell* 80: 661-670) that is covalently coupled to native life-extension polypeptide or life-extension polypeptide fusion proteins, may be performed to determine the life-extension polypeptide binding activity of different agents.

In another embodiment, an *in vitro* screening assay detects agents that compete with another substance (most likely a polypeptide) that binds a life-extension polypeptide. Competitive binding assays are known in the art and need not be described in detail herein. Briefly, such an assay entails measuring the amount of life-extension polypeptide complex formed in the presence of increasing amounts of the putative competitor. For these assays, one of the reactants is labeled using, for example, ^{32}P.

It is also understood that the *in vitro* screening methods of this invention include structural, or rational, drug design, in which the amino acid sequence, three-dimensional atomic structure or other property (or properties) of a life-extension polynucleotide or polypeptide provides a basis for designing an agent which is expected to bind to a life-extension polynucleotide or polypeptide. Generally, the design and/or choice of agents in this context is governed by several parameters, such as the perceived function of the polynucleotide or polypeptide target, its three-dimensional structure (if known or surmised), and other aspects of rational drug design. Techniques of combinatorial

chemistry can also be used to generate numerous permutations of candidate agents. For purposes of this invention, an agent designed and/or obtained by rational drug designed may also be tested in the cell-based assays described below.

Cell-based screening methods

In cell-based screening assays, a living cell containing a functioning life-extension polynucleotide (whether or not an encoding region) is exposed to an agent. The living cell may be in culture or, alternatively, part of a whole, living animal. In contrast (as described above), conventional drug screening assays have typically measured the effect of a test agent on an isolated component, such as an enzyme or other functional protein.

The cell-based screening assays described herein have several advantages over conventional drug screening assays: 1) if an agent must enter a cell to achieve a desired therapeutic effect, a cell-based assay can give an indication as to whether the agent can enter a cell; 2) a cell-based screening assay can identify agents that, in the state in which they are added to the assay system are ineffective to modulate the life-extension polynucleotide and/or polypeptide function, but that are modified by cellular components once inside a cell in such a way that they become effective agents; 3) most importantly, a cell-based assay system allows identification of agents affecting any component of a pathway that ultimately results in characteristics that are associated with life-extension polynucleotide and/or polypeptide function.

In one embodiment, an agent is identified by its ability to modulate, preferably enhance or increase, life-extension polynucleotide and/or polypeptide function in a suitable host cell. A suitable host cell in this context is any host cell which allows such function to be measured. Preferably, the host cell is a nematode cell, either isolated or as part of a whole nematode. Suitable host cells include, but are not limited to, fungi (including yeast), bacterial, insect, mammalian, and amphibian.

In one embodiment, the invention provides methods for identifying an agent that may increase longevity comprising the following steps: (a) contacting at least one agent to be tested with a suitable host cell that has life-extension polynucleotide and/or polypeptide function; and (b) analyzing at least one characteristic which is associated with life-extension polynucleotide and/or polypeptide function in said host cell, wherein an agent is identified by its ability to modulate at least one such characteristic.

Characteristics associated with function of a life-extension polynucleotide and/or polypeptide depend upon the polynucleotide or polypeptide. Functional characteristics include, but are not limited to, transcription, translation (including levels of precursor and/or processed polypeptide), location of protein product (such as membrane localization), any enzymatic activities, such as kinase activity, structural and/or functional phenotypes (such as stress resistance or life cycle), and expression (including repression or de-repression) of any other genes known to be controlled (modulated) by the polynucleotide. Any measurable change in any of these and other parameters indicate that the agent may be useful. Measuring these parameters (such as those using reporter genes) involve methods known in the art and need not be discussed herein. Reporter genes include, but are not limited to, alkaline phosphatase, chloramphenicol acetyl transferase, β-galactosidase, luciferase and green fluorescent protein. Identification methods for the products of reporter genes include, but are not limited to, enzymatic assays and fluorimetric assays. Reporter genes and assays to detect their products are well known in the art and are described, for example in Current Protocols in Molecular Biology, eds. Ausubel et al., Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates. Reporter genes, reporter gene assays and reagent kits are also readily available from commercial sources (Stratagene, Invitrogen and etc.).

Introduction of life-extension polynucleotides depend on the particular host cell used and may be by any of the many methods known in the art, such as microinjection, spheroplasting, electroporation, $CaCl_2$ precipitation, lithium acetate treatment, and lipofectamine treatment.

Polynucleotides introduced into a suitable host cell(s) are polynucleotide constructs comprising a life-extension polynucleotide. These constructs contain elements (i.e., functional sequences) which, upon introduction of the construct, allow expression (i.e., transcription, translation, and post-translational modifications, if any) of life-extension polypeptide amino acid sequence in the host cell. The composition of these elements will depend upon the host cell being used. For introduction into *C. elegans,* polynucleotide constructs will generally contain a selectable marker such as *rol-6 (su1006)* and the life-extension polynucleotide operatively linked to a suitable promoter (when encoding a life-extension polypeptide), such as *tkr-1*. Other suitable host cells and/or whole animals include *Drosophila*, yeast and mammalian cells. Suitable selectable markers for nematode

cells are those that enable the identification of cells that have taken up the nucleic acid, such as morphologic and behavioral markers such as *rol-6* or visual markers such as green fluorescent protein. Screening of the transfectants identifies cells or animals that have taken up and express the polynucleotide.

In some embodiments, a life-extension polynucleotide is operatively linked to an inducible promoter. Use of an inducible promoter provides a means to determine whether the agent is acting via a pathway involving the life-extension polynucleotide. If an agent modulates a functional characteristic of a life-extension polynucleotide and/or polypeptide in a cell in which the inducible promoter is activated, an observation that the agent fails to elicit the same result in a cell in which the inducible promoter is not activated indicates that the agent is affecting at least one step or aspect of life-extension polynucleotide function. Conversely, if the functional characteristic is also observed in a cell in which the inducible promoter is not activated, then it can be assumed that the agent is not necessarily acting solely via the life-extension polynucleotide functional pathway.

Cell-based screening assays of the present invention can be designed, *e.g.*, by constructing cell lines or strains of animals in which the expression of a reporter protein, *i.e.*, an easily assayable protein, such as β-galactosidase, chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) or luciferase, is dependent on life-extension polynucleotide and/or polypeptide function. The cell is exposed to a test agent, and, after a time sufficient to effect β-galactosidase expression and sufficient to allow for depletion of previously expressed β-galactosidase, the cells are assayed for the production of β-galactosidase under standard assaying conditions.

Assay methods generally require comparison to a control sample to which no agent is added. The screening methods described above represent primary screens, designed to detect any agent that may exhibit anti-aging activity. The skilled artisan will recognize that secondary tests will likely be necessary in order to evaluate an agent further. For example, a cytotoxicity assay would be performed as a further corroboration that an agent which tested positive in a primary screen would be suitable for use in living organisms. Any assay for cytotoxicity would be suitable for this purpose, including, for example the MTT assay (Promega).

In some embodiments, a nematode transgenic system (as descibed above) is used for screening. Our discovery that a transgenic nematode comprising *tkr-1* exhibits life

extension, coupled with our discovery that the kinase domain of FGFR can substitute for the kinase domain of FGFR, indicates that our life-extension transgenic nematode system would be especially useful for screening for agents which may (a) exhibit life-extension activity; or (b) exhibit repression of life-extension.

Accordingly, in one embodiment, the invention provides a method for identifying an agent which may modulate life extension, comprising contacting a transgenic nemotode comprising a *tkr-1* polynucleotide with the agent and measuring lifespan. In another embodiment, the transgenic nematode comprises a fusion construct containing a tkr-1 polynucleotide in which the kinase domain of FGFR has been substituted for the kinase domain of tkr-1.

The following examples are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

EXAMPLE 1

Screening for polynucleotides associated with life extension using transgenic C. elegans

Computer similarity search using mammalian stress response receptor genes

A Blast search (Altschol et al.,1990, *J. Mol. Biol.* 215, 403-410) for *C. elegans* genes similar to *c-kit* identified more than seven genes. Two of the most similar open reading frames were C08H9.5 (Blast score 3.7e-62; same as *tkr-1*; see below) and ZK938.5 (Blast score 9.6e-59; same as *tkr-2*; see below). The other identified open reading frames were R151.4 (Blast Score 7.9e-57), F59F3.1 (Blast score 4.5e-53), and F59F3.5 (Blast score 4.1e-51). A homology search for PDGF-R also identified these same *C. elegans* gene members. Therefore, homology searching identified a group of genes similar to *c-kit*/PDGF-R receptors.

A homology search for EGF-R and IGF-R identified let-60/EGF-R and two open reading frames, R151.4 and C01G6.8, respectively. Therefore, the computer search of the *C. elegans* gene database identified genes that were structurally similar to the mammalian stress genes used as a basis for the search.

Construction of transgenics

Although the search method described above effectively identified a pool of stress genes, another method was required to identify particular genes that affect the aging process. The genomic screen identified two cosmids, C11D5 and C08H9, that overlap with each other (Figure 2). To test these sequences to determine whether they conferred stress resistance and/or life extension, we constructed transgenic nematodes.

Strains and Media. The *C. elegans* strains were maintained and handled on nematode growth medium (NGM) agar seeded with *E. coli*, OP50 as a food source (The Biology of Caenorhabditis elegans, Wood, W.B., Cold Spring Harbor Press (1988)). For the stress resistance and life span assays, we used the NGM plates spread with OP50 over the surface (Wood, W.B., supra).

Plasmids. The *p(tkr-1)* plasmid is Bluescript II-based (Stratagene) carrying the 6.5 kb *Spe 1* fragment from the cosmid C08H9 which contains the entire *tkr-1* ORF plus the entire 5' untranslated region plus some of the upstream ORF (total 5' region, 2240 bp). Proc. *Natl. Acad. Sci. USA* (1986) 83:7821-7825; Wilson et al (1994) *Nature* 368: 32-38. The 6.5 kb fragment carrying *tkr-1* also contains a 5' truncated chitanase gene, which should not be expressed because it lacks a promoter region. The region around *tkr-1* contains a tandem array of chitanase genes, these chitanase genes, including truncated forms, have no effect on thermotolerance and lifespan.

The construction of the mutant plasmid, p(*tkr-1* Δkin), was as follows: The *tkr-1* plasmid, digested with *Bg1 II*, was treated with T4 DNA polymerase and was ligated. The resulting plasmid was recovered by *E. coli* transformation. The *tkr-1 Δkin* plasmid has a 4 bp additional sequence (5'-GATC-3') resulting in the frameshift mutation (Figures 2 and 3). The predicted termination codon is located just after the *Bg1 II* site. Primary structure of all plasmids was confirmed by sequencing.

Construction of transgenics. We constructed *C. elegans* transgenics by using a microinjection technique previously described (Mello *et al.*, 1991 *EMBO J.* 10, 3959-3970). A marker plasmid, pRF4 (Mello et al., supra), was used. All transgenic strains were made in the wild-type strain, N2, by microinjecting high copy numbers of each clone together with a plasmid carrying the dominant *rol-6* (*su1006*) marker, that causes a "roller" phenotype in the worms when expressed. All the cosmids and yeast artificial chromosomes (YACs) were obtained from Dr. Alan Coulson, Sanger Center (1994 Nature 368, 32-34).

Each plasmid (0.02 μg/μl) was co-injected with pRF4 (0.2 μl/μg) into the gonads of young adult hermaphrodites. To minimize the possibility that toxic genes may offset the thermotolerance of other genes when over-expressed, we injected a single YAC or cosmid DNA into the worms instead of injecting a pool of DNAs. Transgenics showing either an Age or Str (stress resistance) phenotype were detected by subsequent comparisons with either N2 or control strains carrying only the *rol-6* marker. F2 transgenics carrying the *rol-6* marker were isolated and maintained. More than two transgenics from the independent microinjections were isolated and tested. We did not find any significant effect of the pRF4 on thermotolerance, UV resistance and life span. Integrated *tkr-1* strains were derived from *tkr1-1* or *tkr1-2*. Irradiated Rol offspring (F2) were then backcrossed with the N2 strain five times to obtain stably integrated strains free of mutations produced by the irradiation.

Level of tkr-1 expression in transgenic nematodes. We confirmed that the *tkr-1* transgenics showed high levels of the *tkr-1* RNA and of the *tkr-1* protein, suggesting that *tkr-1* was over-expressed in the transgenic lines. A typical *tkr-1* transgenic line had approximately 20 copies of *tkr-1* as assessed by Southern analysis and overexpressed *tkr-1* mRNA.. The *tkr-2* gene also was over-expressed as RNA in the *tkr-2* transgenics.

Transgenic strains containing only *tkr-1* together with pRF4 (a plasmid carrying the *rol-6(su1006)* dominant behavioral marker) were examined for effects on stress resistance and longevity.

Transgenic screening for a gene conferring stress resistance (thermotolerance and UV resistance) and life extension

Stress resistance assays. For the thermotolerance assay, 4-day-old young adult hermaphrodites were incubated at 35°C. Survival was measured every one or two hours after an initial decline in viability was detected. For the UV resistance assay, the young adults (4 days old) were irradiated with 254 nm UV-C light at 20 J/m^2. Survival was measured every day until all were dead. Each experiment contained two sets of about 20 worms that expressed the marker phenotype. Each experiment was replicated more than twice. The statistical analysis was performed using the Wilcoxon (Gehan) statistic as implemented in the SPSS survival package (SPSS Update, Hull, C.H. and Nie, N.H., McGraw Hill, 1979). Independently isolated transgenics were used in both the thermotolerance and UV assay.

Lifespan assays. The lifespan assay was performed as described previously. Murakami and Johnson, 1996, *Genetics* 143, 1207-1218. Survival of the hermaphrodites was measured every day until they stopped laying eggs, and then measured every few days thereafter.

We constructed an extensive array of either YAC or cosmid transgenics that collectively covered the entire 0.5 Mb region. Both overlapping cosmids C11D5 and C08H9 conferred increased thermotolerance by about 30% (range of 15-60%) in transgenic worms (Figure 3). Transgenics carrying all other cosmids or YACs over the 0.5 Mb region (C03H10, F17E3, K07G10, F07G10, F07E3, R09A5, T10B9, T18C7, ZK1320, W04A3, Y53C12 and Y10G6) had normal thermotolerance (transgenic vs. *rol-6* control $p > 0.1$). As shown in Fig. 8a, the *tkr-1* transgenics are 35% more heat resistant than control transgenics (control-1 and control-2) ($p<.0001$). Survival at 35°C (mean ± SD) was: *control-1*, 654 ± 14 minutes; *control-2*, 682 ± 14 minutes; *tkr-1*, 834 ± 20 minutes; *tkr1-2*, 793 ± 18 minutes. Three additional *tkr-1* transgenics were also thermotolerant. All four *tkr-1* transgenics tested were significantly more thermotolerant by 15% to 30% than wild type (average 20%; $p <0.0001$, typical data are shown in Fig. 8a). No other cosmids or YAC clones conferred such tolerance.

Using subclones, we narrowed the region responsible for the increased thermotolerance to a 6.5 kb fragment containing the common region of the two cosmids and identified a single open reading frame, designated *tkr-1* (tyrosine kinase receptor-1), containing a conceptual receptor tyrosine kinase.

Since a frameshift mutation, *tkr-1 Δkin*, and a truncation mutation, *tkr-1ΔBB*, abolished the thermotolerance phenotype, we concluded that the *tkr-1* gene is responsible for the thermotolerance.

The *tkr-1* gene consists of 10 exons, encoding 502 amino acid residues (Figure 4). The RACE (rapid amplification of cDNA ends) method (Froman *et al.*, supra) confirmed the 5' and 3' ends of the *tkr-1* transcript. The predicted protein sequence has a potential transmembrane domain and shows a strong similarity with the kinase region of the mammalian receptor kinases, *c-kit* (rat *c-kit* identity 35%; similarity 5 %), FGF-R (human FGF-R1; identity 30%; similarity 56%), and PDGF-R (mouse PDGF-R; identity 35%; similarity 56%). The *tkr-1* kinase region has a nucleotide binding site and three potential autophosphorylation sites. The *tkr-1* kinase region has a putative ATP binding site and

three potential autophosphorylation sites (Figure 4). The predicted extracellular domain is only 40 amino acids. Thus, while *tkr-1* may function as a receptor, it may be a receptor with a short extracellular domain or it may form a complex with another membrane protein that contains a longer receptor domain.

tkr-1 is identical to C08H9.5, identified in the computer similarity search of Example 1. This suggests that *tkr-1* is very similar to the *c-kit*/PDGF-R gene family in *C. elegans*. However, the similarity is mainly from the cytoplasmic kinase domain (44% identity against rat *c-kit*) rather than the extracellular ligand binding domain (15% identity against the rat *c-kit*). *tkr-1* lacks the kinase insertion seen in *c-kit*/PDGF-R family between the kinase subdomains, V and VIA. None of the known genes showed similarity to the *tkr-1* extracellular domain. These data suggest that *tkr-1* is a novel tyrosine kinase gene with a similarity to the kinase domain of the *c-kit*/PDGF-R family and FGF-R.

Kinetics of tkr-1 induction after UV and heat stresses. We constructed a green fluorescent protein reporter system, p(tkr-1GFP) and measured fluorescence after UV irradiation or heat shock (Figure 13). The response of transgenic nematodes carrying p(tkr-1-GFP) to UV light (10 or 20 J/m^2) or heat shock (35°C for 2 hours) are shown in Figure 14. The induction of tkr-1 was measured as the intensity of GFP fluorescence. Images of stressed and unstressed transgenic worms were analyzed using the program NIH image. The results show that *tkr-1* is induced upon stress.

UV resistance. We tested the UV resistance of four *tkr-1* transgenics. Two of the transgenics tested showed 30 to 50% more UV resistance than the control transgenics (average 30%; (Figure 8b). The *tkr-1* transgenics were 30 to 50% more resistant to UV irradiation than control transgenics ($p<.0001$). Observed survivals after UV irradiation at 20 J/m^2 (mean ± SD) were: *control-1*, 3.2 ± 0.1 days; *control-2*, 3.2 ± 0.1 days; *tkr-1*, 4.8 ± 0.2 days; *tkr1-2*, 4.6 ± 0.1 days. This suggests that the stress resistance conferred by *tkr-1* is not specific to heat but rather more general and includes other stresses as well. Since multi-stress resistance is a phenotype observed in life-extension mutants in *C. elegans*, the *tkr-1* transgenics share a common phenotype to the life-extension mutants.

Life extension. Four independently isolated *tkr-1* transgenics were tested for their life-extension abilities. The results are shown in Figures 9a and 9b. All of them showed an increase in mean life span ranging from 48 % to 65 % ($p <.0001$). Life expectancies (mean ± SEM) were: (*control-1*) 24.3 ± 1.1 days, (*control-2*) 26.9 ± 1.2 days, (*tkrl-l*) 39.8

± 1.5 days and (*tkrl-2*) 40.2 ± 1.3 days, (tkr2-1) 31.1 ± 1.5 days ($p<0.05$) and (*tkr2-2*) 34.5 ± 1.2 days ($p<0.01$). The life expectancy of the four transgenics was 27.6 days (65% extension; wild type 16.9 days) and their maximum life span was 48 days (77% extension; wild type 27 days) *tkr-1* and the control transgenics, all of which were hermaphrodites, started to produce eggs at 3 days after hatching, suggesting that *tkr-1* does not delay development. Therefore, the *tkr-1* life extension is an extension of adult life span. This life extension is similar to that of the previously reported life-extension mutant, *age-1* (65%), and larger than *spe-26* (30%), and *elk-1* (5-40%).

Effect of various deletions in tkr-1

tkr-1 Δ*BB* is a truncation mutation of *tkr-1* that deletes the whole coding region, except for the portion encoding the first 15 amino acids. This mutation totally abolished thermotolerance (see above), UV resistance and life extension (Table 1). This indicates that the *tkr-1* gene is essential for these phenotypes.

tkr-1 Δ*kin* is a frameshift mutation in *tkr-1* that deletes the cytoplasmic portion of the encoded polypeptide, including the kinase domain, with a small C-terminal domain (*tkr-1* Δ*kin*; *see* Fig. 2 and Fig. 3). *tkr-1* Δ*kin* abolished the thermotolerance, the UV resistance, and the life extension observed in the wild type *tkr-1* (Table 1). This indicates that the cytoplasmic kinase domain plays a major role in the stress resistance and life extension. In addition, the mutant *tkr-1* Δ*kin* can still confer a moderate life extension, suggesting that something other than the kinase domain also plays a minor role in the increased longevity, but not in the stress resistance.

The results of a typical experiment are shown in Table 1. More comprehensive data is summarized in Table 2. All experiments were replicated. The *tkr-1* transgenics in each experiment were significantly different from the control transgenics ($p<.0001$), whereas the *tkr-1* Δ*kin* transgenics were indistinguishable from the controls ($p>0.2$) except for the life span experiments ($p<.0001$). The control transgenics carried only a marker plasmid, pRF4, whereas the *tkr-1* and *tkr-1* Δ*kin* transgenics carried the marker plasmid and the *tkr-1* plasmid and the *tkr-1* Δ*kin* plasmid, respectively.

Table 1.

Effect of deleting the *tkr-1* cytoplasmic domain.

Transgenic	Heat % increase (mins.)	N	UV % increase (days	N	Life span % increase (days)	N
Control	0 (625)	152	0 (3.1)	198	0 (16.9)	123
Tkr-1	19.6 (750)	88	36.2 (4.1)	220	63.2 (27.6)	147
tkr-1 Δ*kin*	5.2 (636)	158	2.6 (3.1)	208	7.1 (18.1)	150
Tkr-1 Δ*BB*	1.0 (631)	70	0 (3.1)	99	5.3 (17.8)	66

Table 2.

Effect of the *tkr-1, tkr-2, and tkr-1* derivatives

Strain		Life Expectancy		UV Resistance		Thermotolerance	
		Mean ± SEM (days)	(N)	Mean ± SEM (days)	(N)	Mean ± SEM (minutes)	(N)
1.	control	25.6 ± 1.3	(123)	3.5 ± 0.2	(198)	625 ± 3	(152)
2.	*tkr*-1	42.3 ± 2.1	(147)	4.5 ± 0.1	(220)	750 ± 1	(88)
3.	*tkr*-2	30.4± 3.6	(98)	3.7 ± 0.2	(258)	656 ± 16	(342)
4.	control	16.3 ± 5.0	(98)	2.9 ± 0.3	(102)	627 ± 10	(99)
5.	*tkr1*ΔBB	17.8 ± 2.2	(132)	3.1 ± 0.1	(154)	631 ± 3	(70)
6.	control	16.9 + 0.1	(86)	3.2 + 0.1	(123)	623 + 4	(112)
7.	*tkr1*Δkin	16.4 + 1.2	(74)	3.5 + 0.2	(124)	624 + 2	(158)
8.	*tkr-FGFR1*	28.3 + 0.3	(108)	4.1 + 0.1	(169)	744 + 14	(106)
9.	control	17.6 + 0.4	(96)	3.2 + 0.1	(113)	664 + 10	(119)
10.	*tkr*-1int	28.3 + 4.1	(102)	4.7 + 0.3	(56)	836 + 32	(52)

11.	control	16.0 + 3.3	(80)	2.9 + 0.5	(71)	558 + 2	(67)
12.	*daf*-16 (m26)	15.1 + 1.6	(94)	3.0 + 0.9	(58)	530 + 10	(48)
13.	*tkr*-1	24.4 + 2.9	(94)	4.7 + 0.4	(58)	863 + 3	(53)
14.	*tkr*-1; *daf*-16(m26)	15.8 + 3.0	(61)	3.5 + 0.9	(47)	644 + 5	(95)

Tkr-1 transgenic strains were further characterized to determine whether they display phenotypic alterations observed in other Age mutants, such as reduced fertility (*spe-26*), altered developmental timing (*Clk* mutants), or constitutive dauer formation (*age-1* and *daf-2*). The *tkr-1* transgenic strains have normal fertility (mean fertility +/- SEM 303 +/- 12 for *tkr-1* transgenics and 298 +/-14 for the wild type control) and a normal time of fertility (mean +/- SEM 73.0 +/- 3.8 hours after hatching for *tkr-1* transgenics and 72.0 +/- 4.3 hours for the wild-type control). Unlike *age-1* and *daf-2*, *tkr-1* transgenic strains do not form dauers at 27°C when well fed; instead, they appear wild type with regard to dauer formation. Thus, the effects of *tkr-1* are specific to the adult phase and show no other phenotypes previously ascribed to other Age mutants.

EXAMPLE 2

Further characterization of tkr-1 and mapping of clustered tkr-1 family members

Determination of the *tkr-1* transcriptional start and end

The RACE method was used as described (Froman et al., 1988 *Proc. Natl. Acad. Sci. USA* 85, 8998). The first strand was synthesized using a reverse transcriptase system (LifeTechnologies). Five µg of total RNA from a mixed population of the worms was incubated with AP primer for 10 minutes at 70°C in RNase free water. The mixture was incubated at 42°C for 2 minutes in buffer (20 mM Tris-HCl pH 8.4, 50 mM $MgCl_2$, 2.5 mM $MgCl_2$, 10 mM DTT, 500 nM AP primer, 2.0 mM dNTP). Reverse transcriptase (Life Technologies) was added to the reaction mixture, incubated at 42°C for 50 minutes, and inactivated at 70°C for 15 minutes. The resulting RNA/DNA hybrids were treated with RNase H. We then amplified the 3' or 5' end of *tkr-1* or *tkr-2* by using the first primer pair. The resulting PCR product was used to amplify the same region using the second set of the primers (nested PCR). The PCR reaction conditions were 95°C for 10 minutes, 35 cycles of both 95°C for 30 sec and 65°C for 30 sec, and 72°C for 10 minutes using a Taq

polymerase (Perkin Elmer). The sequences of the primers used are: AP (5'-GGC CAC GCG TCG ACT AGT AGT ACT-$(T)_{12-15}$-3') (SEQ ID NO:1), AUAP (5'-GGC CAC GCG TCG ACT AGT AGT ACT-3') (SEQ ID NO:2), tkl-F1 (5'-CCG GAT GTA ATT GGG TAG GAG TTG G-3') (SEQ ID NO:3), tk1-F2 (5'-GTT GGC TCA AGA AGA TAC TCA TCA CC-3') (SEQ ID NO:4), tk1-B2 (5'-CCG CGT TTA GTG AAA GAG GCC TAC-3') (SEQ ID NO:5), tk-B3 (5'-CGT CAG ATT CTT ACA ACA TG-3') (SEQ ID NO:6), tk2-F1 (CCG GAA GTA ATT GGA TAA GGC-3') (SEQ ID NO:7), tk2-F3 (5'-TAA ATC AAC AAG GCC CAA TGA CGG-3') (SEQ ID NO:8), tk2-B4 (5'-CAT TGG GCC TTG ATT TAG T-3') (SEQ ID NO:9), SL1 (5'-GGT TTA ATT ACC CAA GTT TGA G-3') (SEQ ID NO:10), and SL2 (5'-GGT TTT AAC CCA GTT ACT CAA G-3') (SEQ ID NO:11). The first and second primer pairs used were: for the 3' end of *tkr-1* (AUAP and tk1-B; AUAP and tk-B3), for the 3' end of *tkr-2* (AUAP and tk2-B4; AUAP and tk-B3), for the 5' end of *tkr-1* (SLI and tk1-FI; SL1 and tkl-F2), and for the 5' end of *tkr-2* (SL1 and tk2-B3; SL1 and tk2-F1). The primer for SL2 was also used instead of the SL1 primer as a control. The PCR products were sequenced to determine the ends.

Mapping tkr-1 family members

We mapped the *tkr-1* family members. Map positions of all the sequenced cosmids were determined in the *C. elegans* genome project (Wilson et al., supra). The members formed tandem repeats scattered in three positions (Figure 10). One group, including *tkr-1*, *tkr-2* and C08H9.8, locates to the left of *unc-4* on chromosome II. The direction of the predicted transcription is the same in all three. F59F3.5 and F59F3.1 also form a tandem repeat near *daf-12* on chromosome X. Tandem repeats of *kin-15* and *kin-16* were previously reported and are localized on chromosome II. It has been shown that this *kin-15* and *kin-16* pair transcribe in a single transcription unit. Since the intervals between the other members are much larger (from 2 to 13 kb) than the *kin-15* pair (300 bp), it is not clear whether the other members are in the same transcription unit.

EXAMPLE 3

The kinase domain of a human receptor substitutes for the kinase domain of tkr-1 in *C. elegans*

The kinase domain of *tkr-1* shows similarity to the mammalian receptor kinases, *FGF-R*, *c-kit* and *PDGF-R*. The *tkr-1* kinase domain is similar to that of *FGF-R* in that they lack regulatory sites seen in the KI domains of *c-kit* and *PDGF-R*. Since the kinase domain of the *c-kit.PDGF-R* family may interact with additional signaling proteins at the KI domain, we chose one of the *FGF-R* family members, human *FGF-R1*, and tested a functional conservation of the cytoplasmic kinase domain between them.

We constructed a chimeric *tkr-1* gene, *tkrl-FGFR*, and substituted its kinase domain with that of the *FGF-R1*. The chimeric plasmid, p(tkrl-FGFR), was constructed as follows: *p(tkr-1)* was digested with *Bgl II*, treated with T4 DNA polymerase to make it a blunt ended, desalted and concentrated. The plasmid was then digested with *Xba 1*. The linearized plasmid was ligated with a *Fsp 1/Xba 1* fragment from a plasmid carrying the human *FGF-R1* kinase domain (Wennstrom et al., 1991 Growth Factors 4, 197-208). This resulted in the *tkr-1* and human FGF-R fusion gene substituting the *tkr-1* cytoplasmic kinase domain for the FGF-R kinase domain. The primary DNA structure of all the mutant and the fusion constructs was confirmed by sequencing.

As shown in Figure 11c, the chimeric *tkrl-FGFR* conferred an increased life expectancy by 68%, resistance to heat by 19% and UV light by 32% (Table 2 and Fig. 11c). Survivals (mean ± SD.) at 35°C were: (*control-1*) 627 ± 91 minutes, (*control-2*) 619 ± 87 minutes, (tkr1-FGFR-1) 730 ± 86 minutes, (*tkr-l-FGFR-2*) 758 ± 86 minutes, (*tkr1 Δkin-1*) 623 ± 93 minutes (p=0.8), (*tkr1 Δkin-2*) 626 ± 93 minutes (p=0.7). Survival after UV irradiation at 2O J/m^2 (mean ± SD) were: (control-1) 3.3 ± 0.9 days, (control-2) 3.1 ± 0.7 days, (*tkr1-FGFR-1*) 4.3 ± 1.0 days, (*tkrl-FGFR-2*) 4.2 ± 1.1 days, (*tkr1 Δkin-l*) 3.3 ± 0.8days (p>0.1), (*tkr1 Δkin-2*) 3.0 ± 0.8days (p>0.1). Life expectancies (mean ± SD) were: (control-1) 16.8 ± 6.4 days, (control-2) 16.9 ± 5.7 days, (*tkrl-FGFR-1*) 28.7 ± 9.6 days, (*tkrl-FGFR-2*) 28.0 ± 11.1 days, (*tkr1 Δkin-l*) 15.2 ± 7.4 days (p=0.4), (*tkr1 Δkin-2*) 17.5 ± 6.9 days (p=0.6). We also confirmed that the chimeric gene was over-expressed at the RNA level. These data suggest that the kinase domain of *tkr-1* can be substituted with that of human FGFR1. This conservation between *tkr-1* and the human receptor kinase raises

the possibility that they share a downstream signaling pathway for the stress resistance and increased longevity.

EXAMPLE 4

tkr-1 homolog, tkr-2, shows a weaker longevity function

We searched *tkr-1* family members in *C. elegans* using a Blast search (Fig. 5a-c). We identified the best *C. elegans tkr-1* homolog, termed *tkr-2* (tyrosine kinase receptor-2; >90% identity). Other members that were identified are less similar to *tkr-1*; *kin-15* (identity 40%) *kin-16* (identity 47%), F59F3.5, F59F3.1, MO1B2.e and R09D1.13.

tkr-2 encodes a 498 amino acid protein composed of 9 exons and is nearly identical to *tkr-1* protein amino acid sequence. The *tkr-2* gene is located in cosmid F17E3, near *tkr-1*. The primary structures of *tkr-1*, *tkr-2* and FGFR-1 are shown in Figure 6. *tkr-1* and *tkr-2* are 91% identical and 93% similar in the kinase domain.

We tested *tkr-2* to see whether it could confer stress resistance and increases in life span like *tkr-1*. Plasmid *ptkr-2* (accession number: z49918;ZK938.5) is a Bluescript-based plasmid that carries a 4.1 kb EcoRI fragment from F17E3, containing the entire *tkr-2* ORF plus the entire 5' untranslated region plus some of the upstream ORF (total 5' region, 1245 bp). The primary structure was confirmed by sequencing.

The *tkr-2* transgenics showed no detectable difference in resistance to heat and UV light (Figure 8) and a weak but statistically significant ($p < 0.05$) extension of life span (Figure 9). This suggests that stress resistance and life extension is rather specific to *tkr-1* but not a general feature of the receptor kinase genes similar to *tkr-1*.

While exemplary preferred embodiments of the present invention are described herein with particularity, those having ordinary skill in the art will recognize various changes, modifications, additions, and applications other than those specifically described herein, and may adapt the preferred embodiment and methods without departing from the spirit of the invention.

CLAIMS

What is claimed is:

1. A method of identifying a polynucleotide associated with longevity, comprising measuring lifespan of a transgenic nematode comprising the polynucleotide, wherein the polynucleotide is identified by its ability to increase the lifespan of the transgenic nematode compared to a suitable control.

2. The method of claim 1, wherein the nematode is *C. elegans.*

3. The method of claim 1, wherein the polynucleotide is associated with stress response.

4. The method of claim 3, wherein the polynucleotide encodes a receptor.

5. The method of claim 4, wherein the receptor is a kinase receptor.

6. The method of claim 3, wherein the polynucleotide is selected from the group consisting of Jun N-terminal kinase, p38/HOG-1, ELK1, CL100, C-Jun, c-kit, platelet derived growth factor receptors, insulin-like growth factor receptor, epidermal growth and factor receptor.

7. A method of identifying a polynucleotide associated with longevity, said method comprising a) conducting a computer similarity search for a polynucleotide suspected to be involved in stress resistance and b) measuring lifespan of a transgenic nematode comprising the polynucleotide identified in step (a), wherein the polynucleotide is associated with life extension if expression of the polynucleotide confers longer lifespan than a suitable control.

8. A method of identifying an agent that may modulate life-extension comprising contacting a transfected cell comprising a polynucleotide identified in claim 1

or a polypeptide encoded by the polynucleotide identified in claim 1 with the agent, wherein the agent is identified by its ability to modulate a functional characteristic of a life-extension polynucleotide or polypeptide.

9. The method of claim 8, wherein the polynucleotide comprises a region of at least 15 contiguous nucleotides of SEQ ID NO:2.

10. The method of claim 8, wherein the polypeptide comprises a region of at least 10 contiguous amino acids of SEQ ID NO:3.

11. An in vitro method for screening for an agent that may modulate life extension said method comprising the steps of:

a) contacting the agent to be tested with a polynucleotide identified in claim 1 or a polypeptide encoded by the polynucleotide identified in claim 1; and

b) measuring a functional characteristic of said polynucleotide or polypeptide, wherein the agent is identified by its ability to modulate said functional characteristic.

12. The method of claim 11, wherein the functional characteristic is transcription.

13. The method of claim 11, wherein the functional characteristic is translation.

14. The method of claim 11, wherein the functional characteristic is binding to a chemical moiety.

15. The method of claim 11, wherein the functional characteristic is enzymatic activity.

16. The method of claim 15, wherein the enzymatic activity is kinase activity.

17. The method of claim 11, wherein the polynucleotide comprises a region of at least 15 contiguous nucleotides of SEQ ID NO:12.

18. The method of claim 11, wherein the polypeptide comprises a region of at least 10 contiguous amino acids of SEQ ID NO:13.

19. A method for screening for an agent that may modulate life extension; said method comprising the steps of:

a) contacting the agent to be tested with a transgenic nematode comprising a *tkr-1* polynucleotide; and

b) measuring lifespan of the nematode, wherein the agent is identified by its ability to modulate lifespan compared to the absence of the agent.

20. The method of claim 19, wherein the *tkr-1* polynucleotide comprises a kinase domain from fibroblast growth factor receptor, said kinase domain replacing a kinase domain of *tkr-1*.

21. The method of claim 20, wherein the kinase domain is from human FGF-R1.

22. A method for screening for an agent that may modulate life extension, said method comprising the steps of:

a) contacting the agent to be tested with a transgenic nematode comprising a polynucleotide encoding the kinase domain of human fibroblast growth factor receptor; and

b) measuring a functional characteristic of said kinase domain, wherein the agent is identified by its ability to modulate said functional characteristic.

23. The method of claim 22, wherein the functional characteristic is kinase activity.

24. A transgenic nematode containing a chimeric *tkr-1* gene comprised of a human FGF-R1 receptor kinase domain.

25. A chimeric nematode *tkr-1* gene comprised of a human FGF-R1 receptor kinase domain.

26. A transgenic nematode comprising a *tkr-1* transgene.

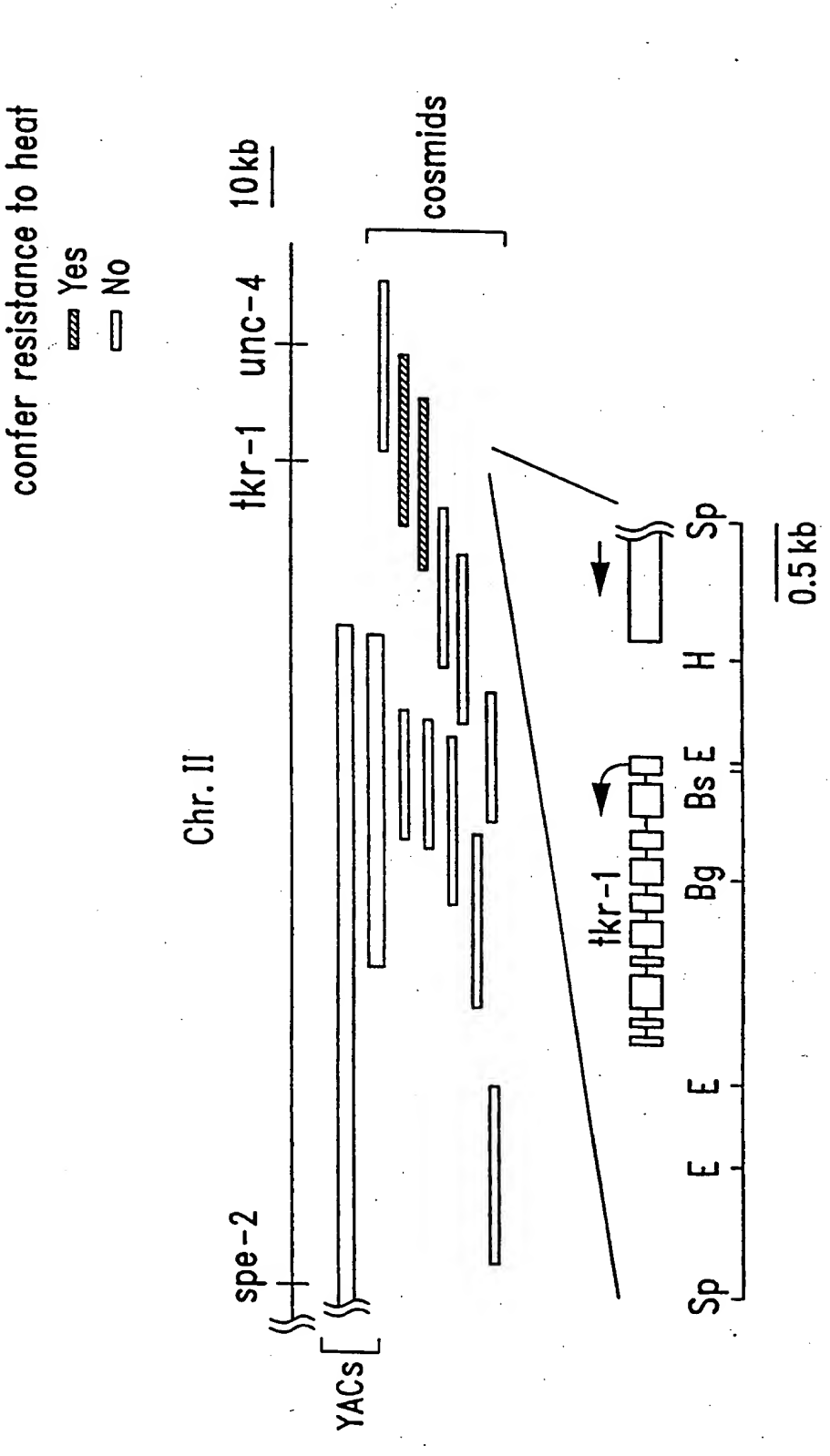
confer resistance to heat
Yes
No
10 kb
cosmids
tkr-1
unc-4
Chr. II
spe-2
YACs
tkr-1
Sp
H
Bs E
Bg
E
E
Sp
0.5 kb

Fig. 1

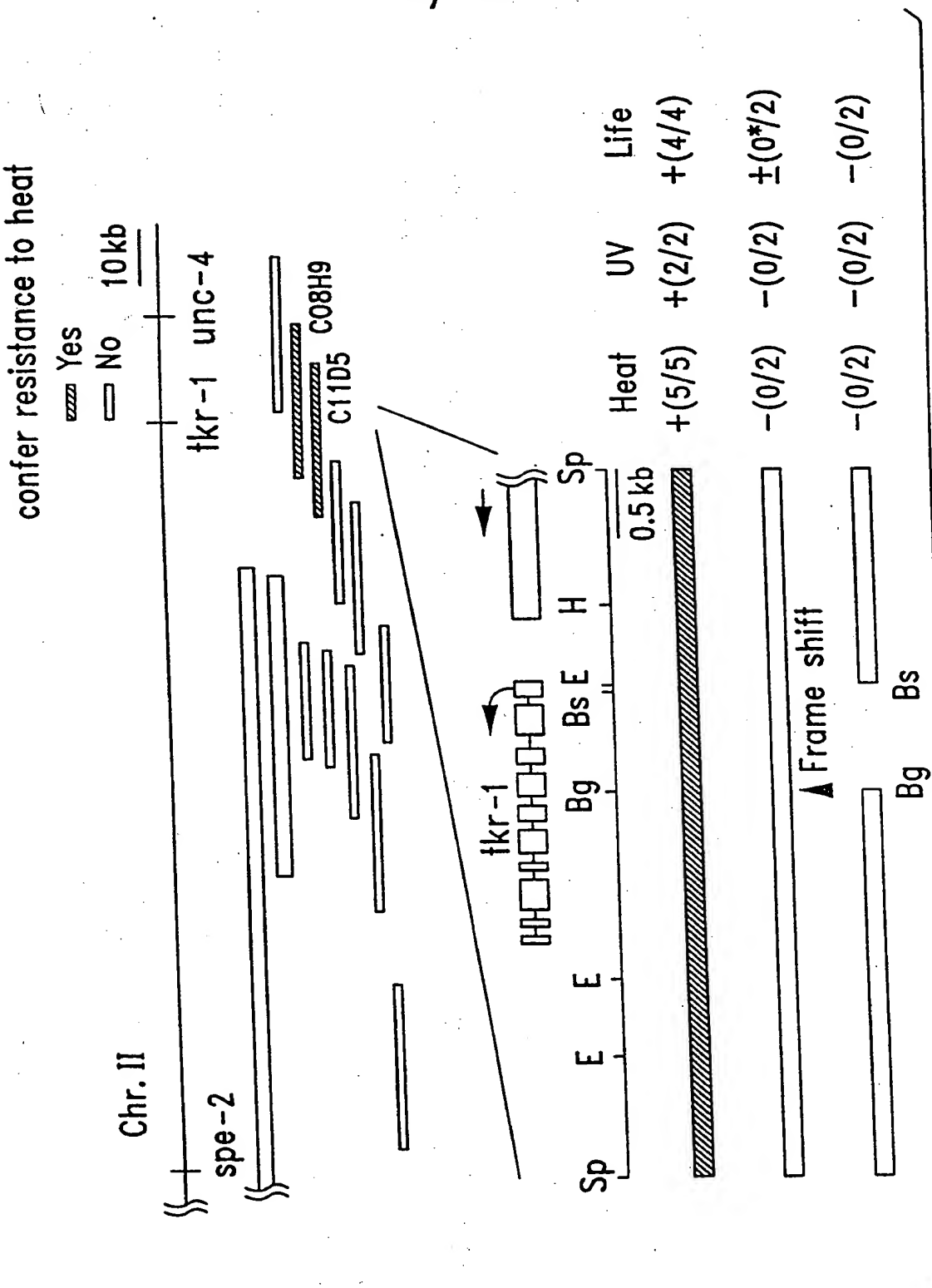

Fig. 2

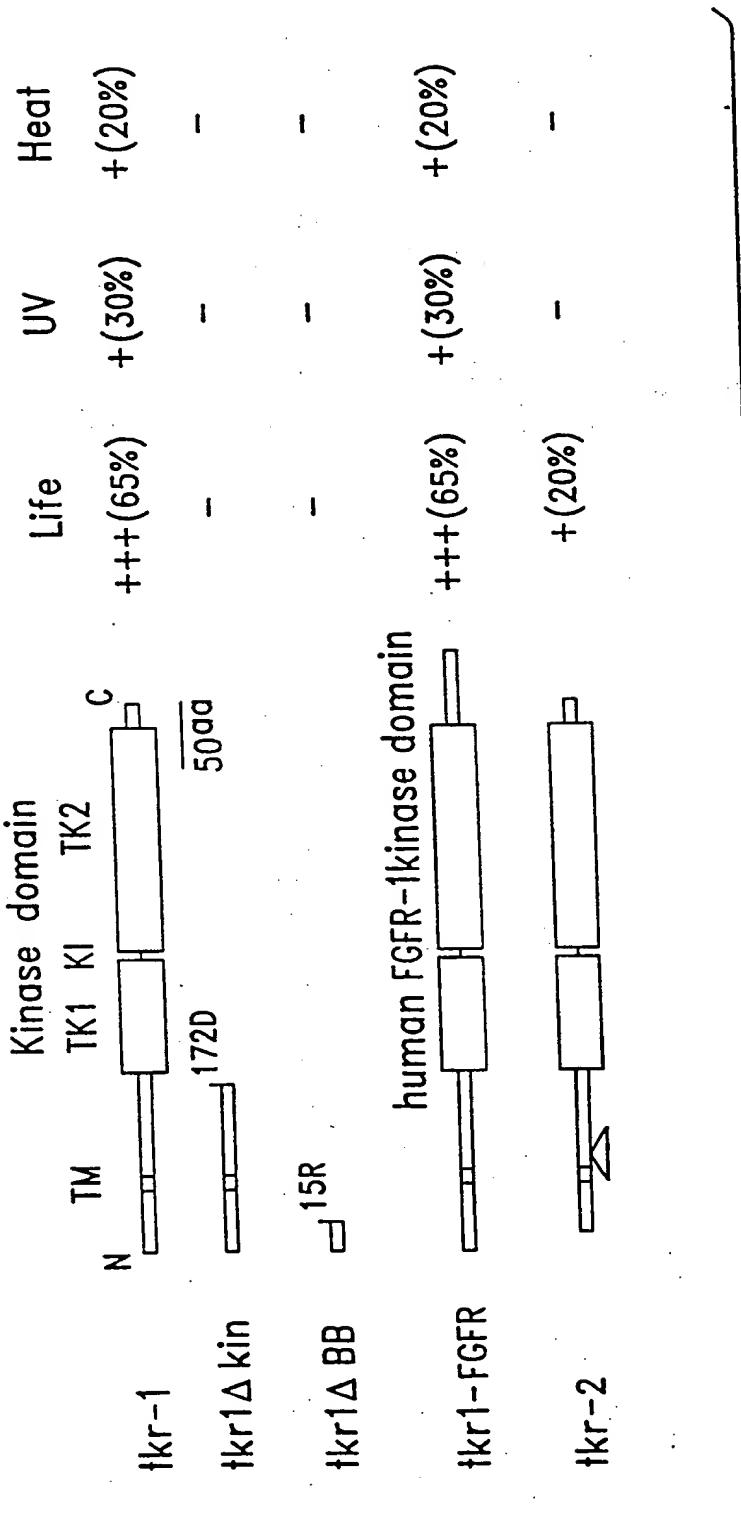
Kinase domain
TM
TK1
KI
TK2
C
N
50aa
172D
15R
human FGFR-1kinase domain
tkr-1
tkr1Δ kin
tkr1Δ BB
tkr1-FGFR
tkr-2
Life
UV
Heat
+++(65%)
+(30%)
+(20%)
-
-
-
-
-
-
+++(65%)
+(30%)
+(20%)
+(20%)
-
-

Fig. 3

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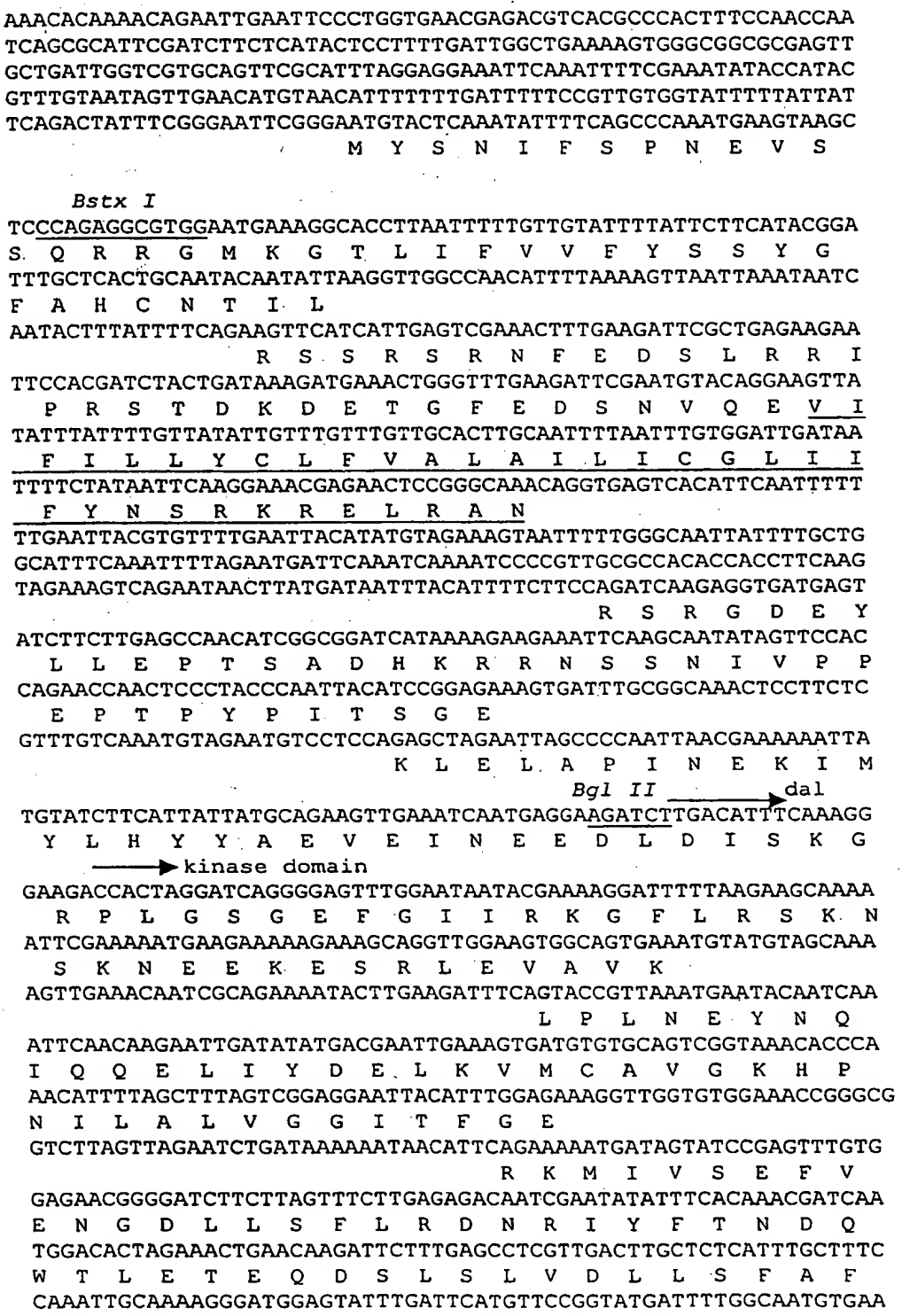
Fig. 4



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```
Q  I  A  K  G  M  E  Y  L  I  H  V  P
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                  C  V  H  R  D  L  A  L  R  N  V  L  I  K
AGAAAAATCGAATAATTCGAATTGCAGACTTTGGATTGGCTAGGAGGCATAAAAATAAAG
   K  N  R  I  R  I  A  D  F  G  L  A  R  R  H  K  N  K  D
ATTATTACAAGTTAGTTCTTTTTATTCAAAAGCTCAAGAGCGTTTCTAGTGGTAAATTTA
   Y  Y
AAAAACGTTTAACTATAAAAATACAGAACTCAAAGTGTCGATACACCGCTTCCAATACAC
                        K  T  Q  S  V  D  T  P  L  P  I  H
TGGATGGCACCTGAAAGTATAGACAAATTACTGTTCACTCAGAAATCAGATGTTTGGTCA
W  M  A  P  E  S  I  D  K  L  L  F  T  Q  K  S  D  V  W  S
TATGGGGTCTGTCTTTATGAACTATTCTCTTTGGGAAAATCACCTTATGAAAATGTGATA
Y  G  V  C  L  Y  E  L  F  S  L  G  K  S  P  Y  E  N  V  I
AAATATGATCAAAGAGATTTTTATTGGAAGTATGTGCTGTCTTACTTGAATGAAGGGAA
K  Y  D  Q  R  D  F  Y  W  K  Y  V  L  S  Y  L  N  E  G  K
AGACTTGCACAGCCTGCACATGCTGACGCAGAAATGTAAGTTGAAAAAGTATTTTACACT
R  L  A  Q  P  A  H  A  D  A  E
ACTAAAATATCACCAATTACAGATATAATGTAATGAAACTATGCTGGGATCTAGATATGA
                     I  Y  N  V  M  K  L  C  W  D  L  D  M  N
ATAGTCGTACAACATTCTTAGACTGCATTGAGTTTTTTGAGAAAGAATTGAAAACAACAT
   S  R  T  T  F  L  D  C  I  E  F  F  E  K  E  L  K  T  T  S
CAAATGAGGTAAGTGACATTTTGAAATGTTTTGGTTGGAACTTTCATTGTACTGTCTTTC
   N  E
AGTATTTTCTTGATCTCACCAGAAAGCTCCGTTCAGAAACCAACAATCAACTTCGACTTA
   Y  F  L  D  L  T  R  K  L  R  S  E  T  N  N  Q  L  R  L  S
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   N  W  L  S  D  E  K  H  C  D  S
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TTCCTCTTCCTCAGTTTTATTTTTCTTATTTCACGTTTTTCCTTTAACACATCATCTCTC
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```

Fig. 4-1

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Fig. 5(a)

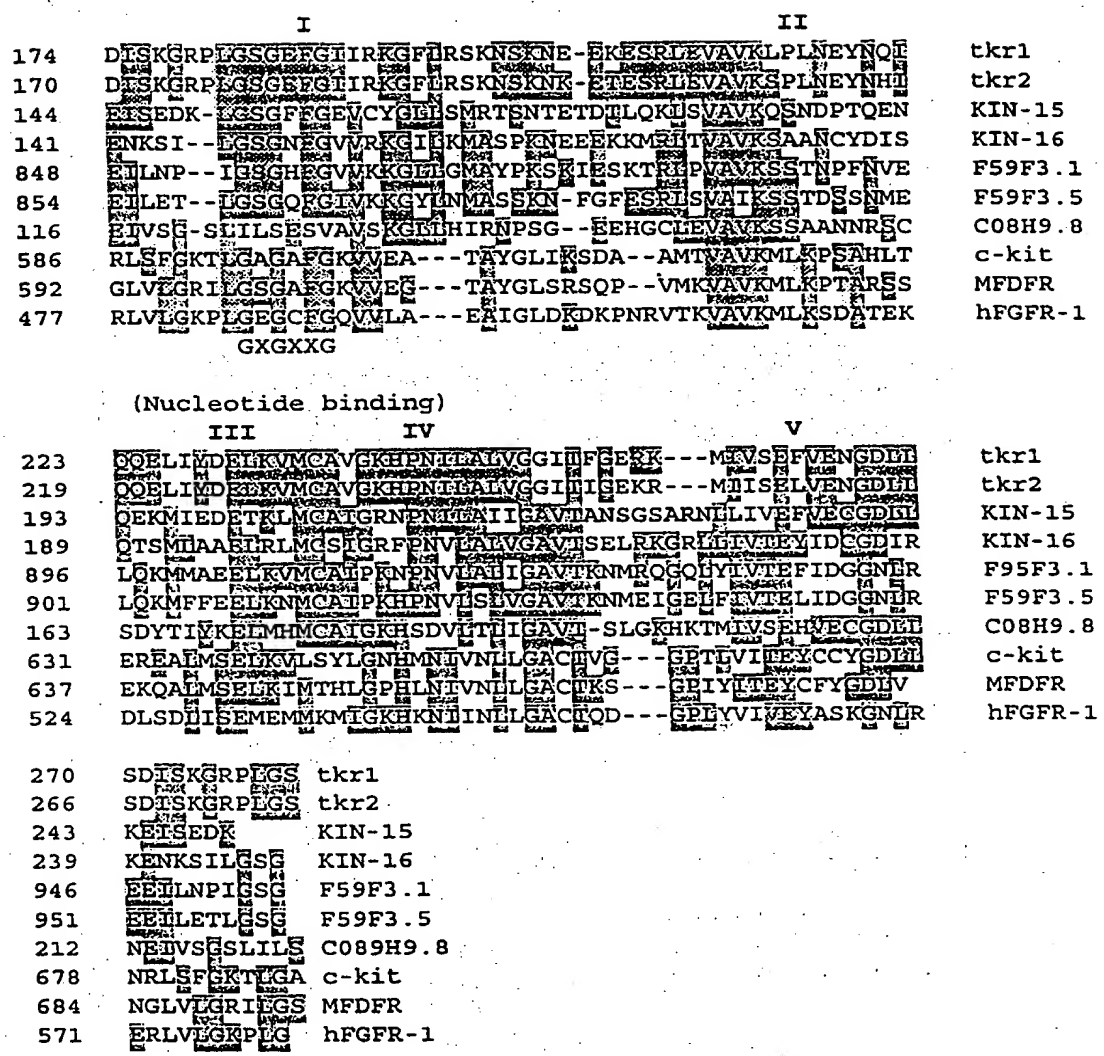

Fig. 5(b)

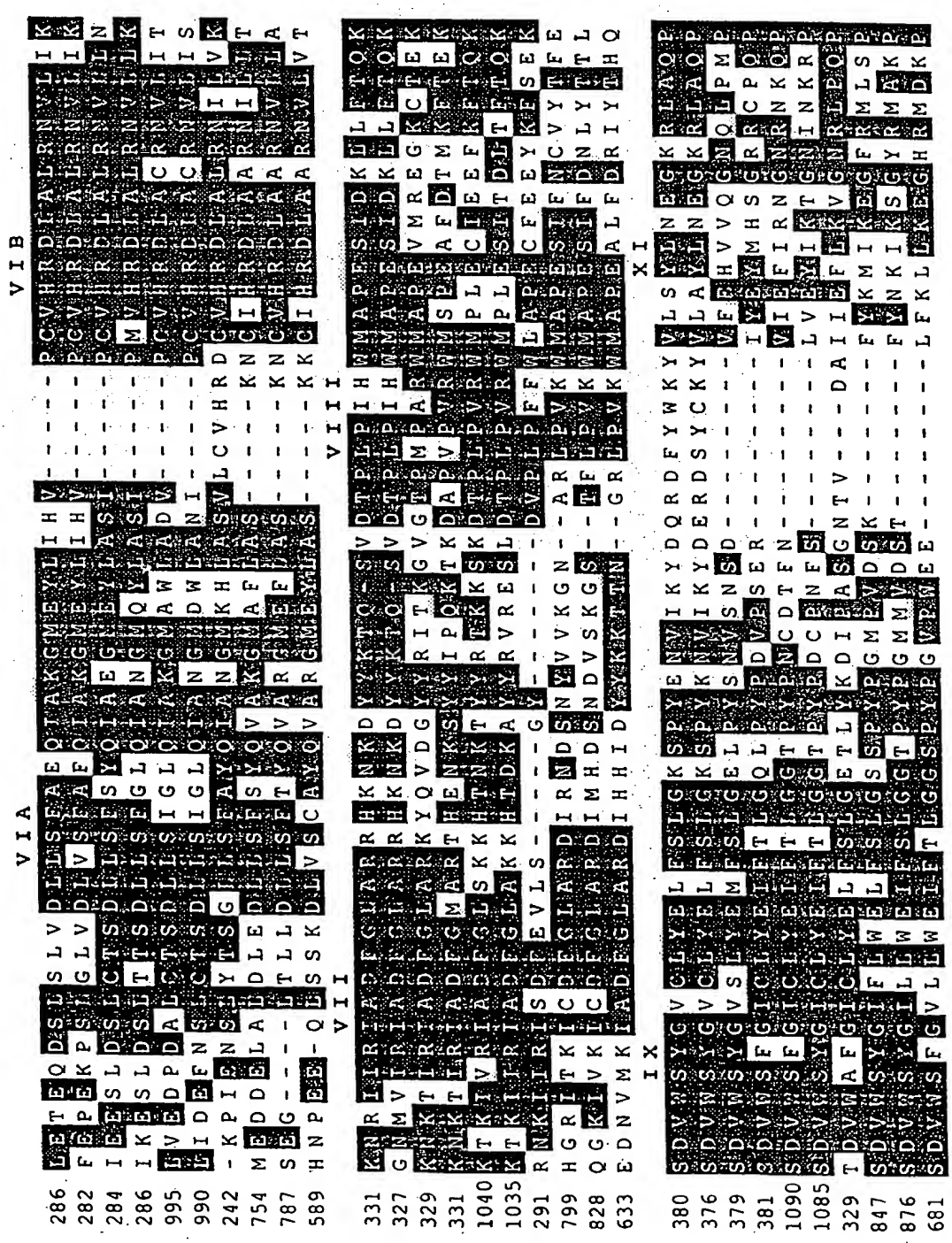

Fig. 5(c)

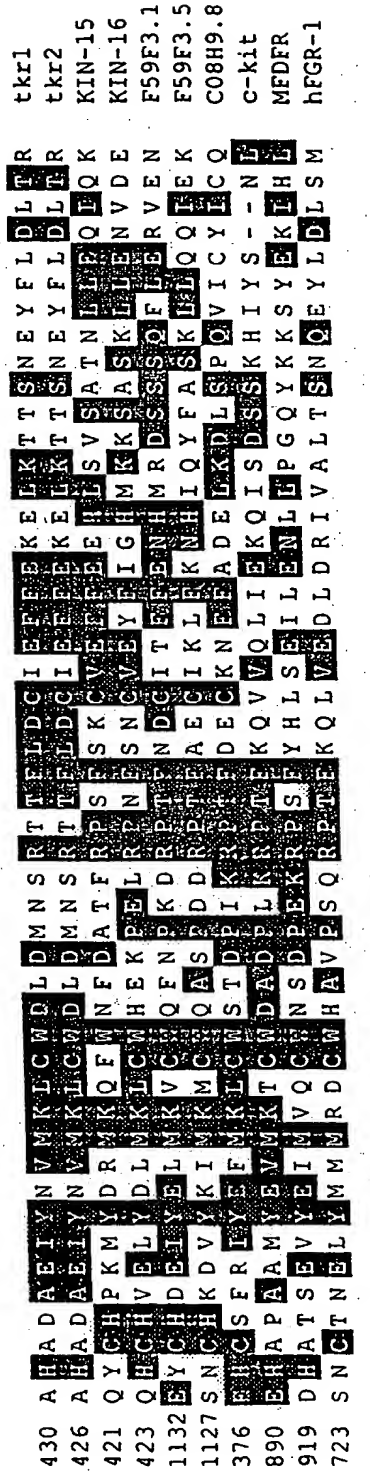

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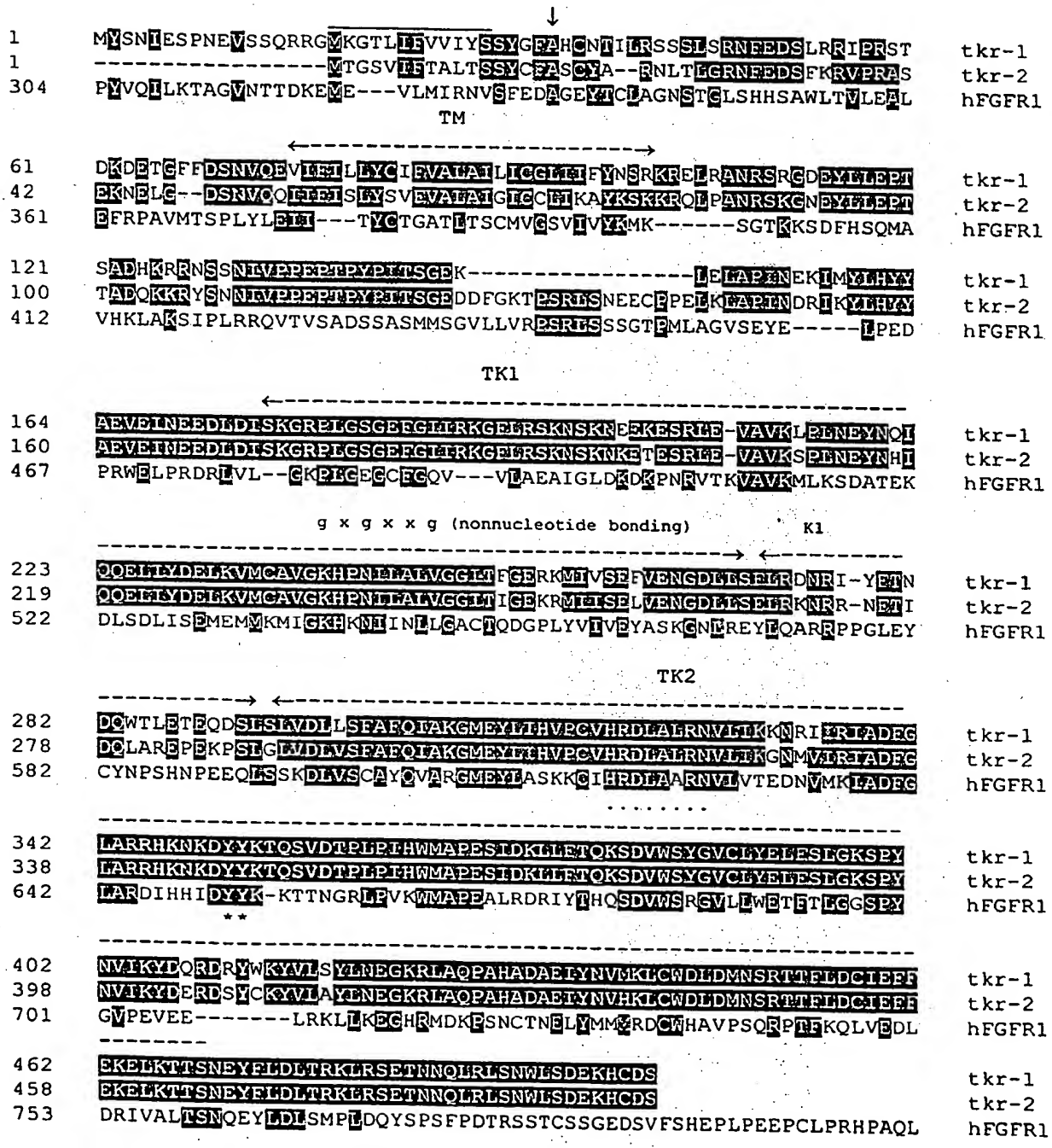
Fig. 6


TM
TK1
g x g x x g (nonnucleotide bonding)
K1
TK2
tkr-1
tkr-2
hFGFR1

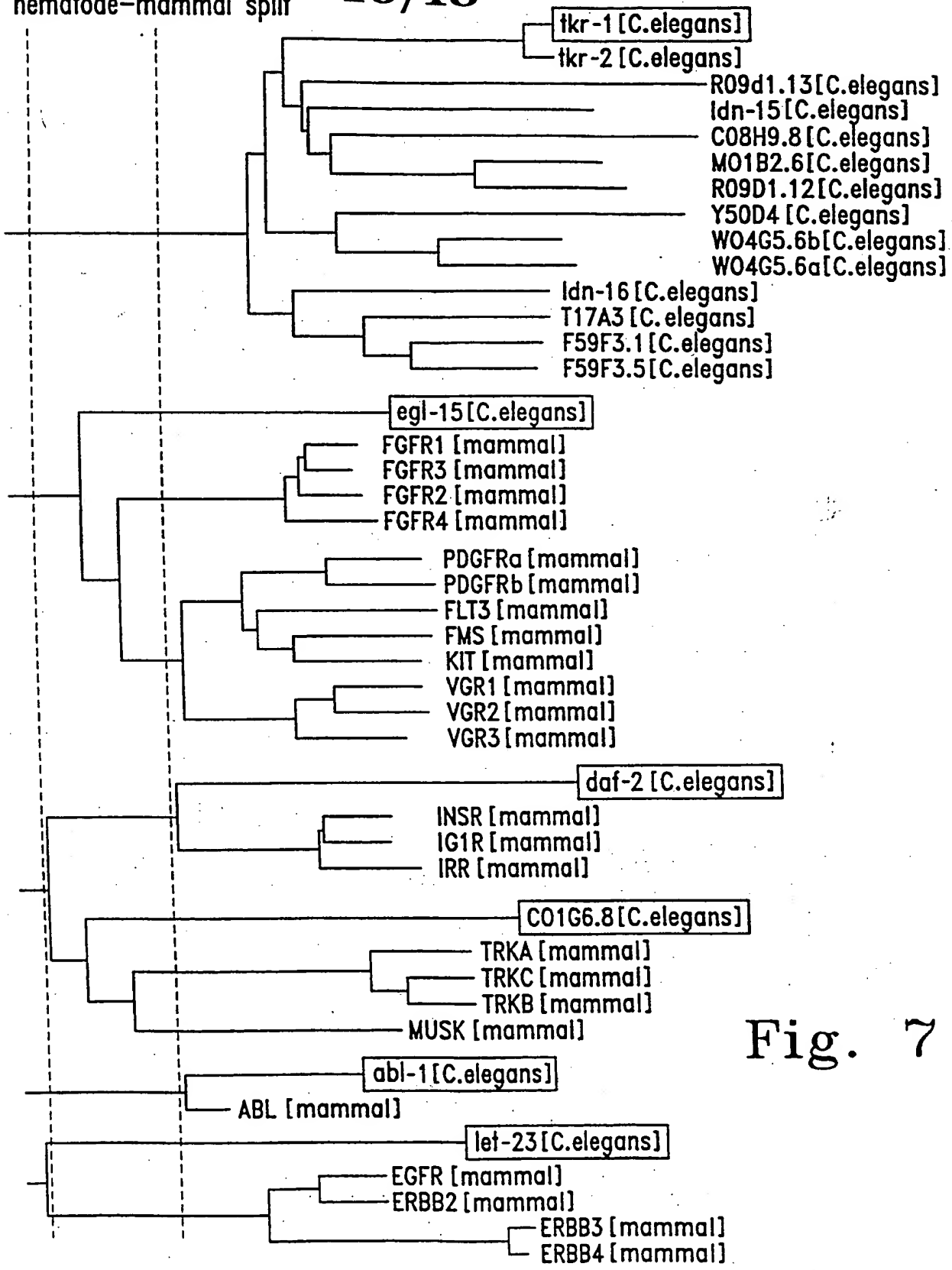
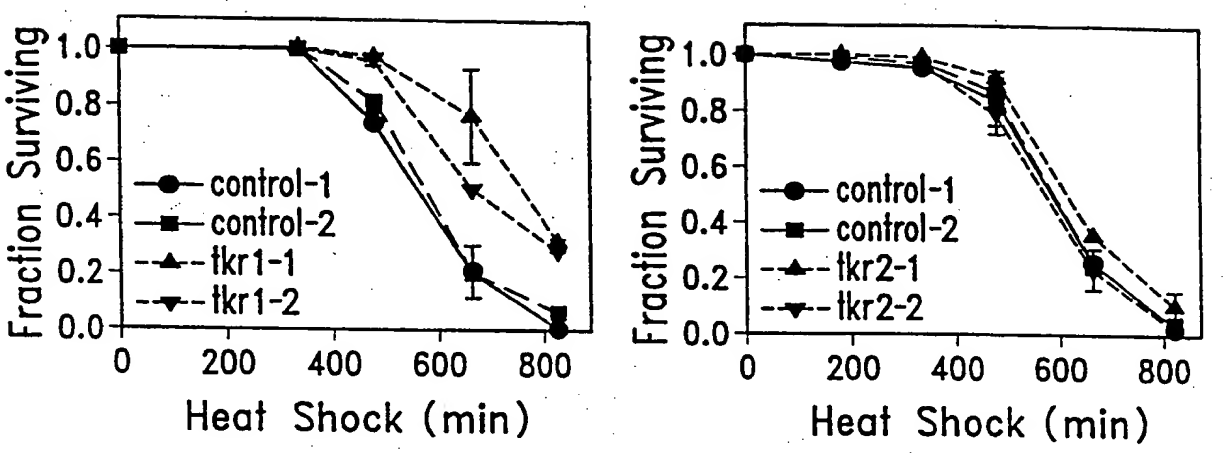


Fig. 7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1.0
0.8
0.6
0.4
0.2
0.0
control-1
control-2
tkr1-1
tkr1-2
0
200
400
600
800
Heat Shock (min)
Fraction Surviving
1.0
0.8
0.6
0.4
0.2
0.0
control-1
control-2
tkr2-1
tkr2-2
0
200
400
600
800
Heat Shock (min)

Fig. 8a

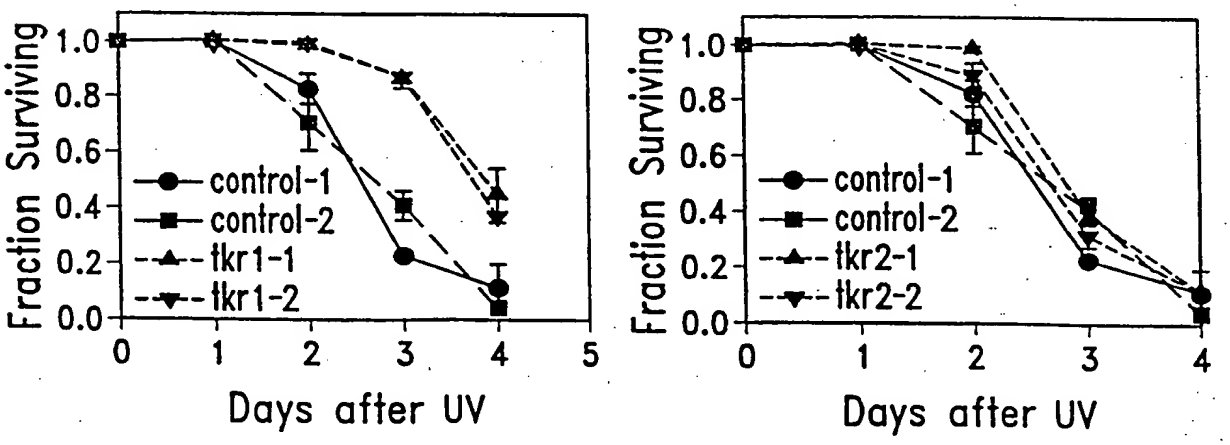
Fraction Surviving
1.0
0.8
0.6
0.4
0.2
0.0
control-1
control-2
tkr1-1
tkr1-2
0
1
2
3
4
5
Days after UV
Fraction Surviving
1.0
0.8
0.6
0.4
0.2
0.0
control-1
control-2
tkr2-1
tkr2-2
0
1
2
3
4
Days after UV

Fig. 8b

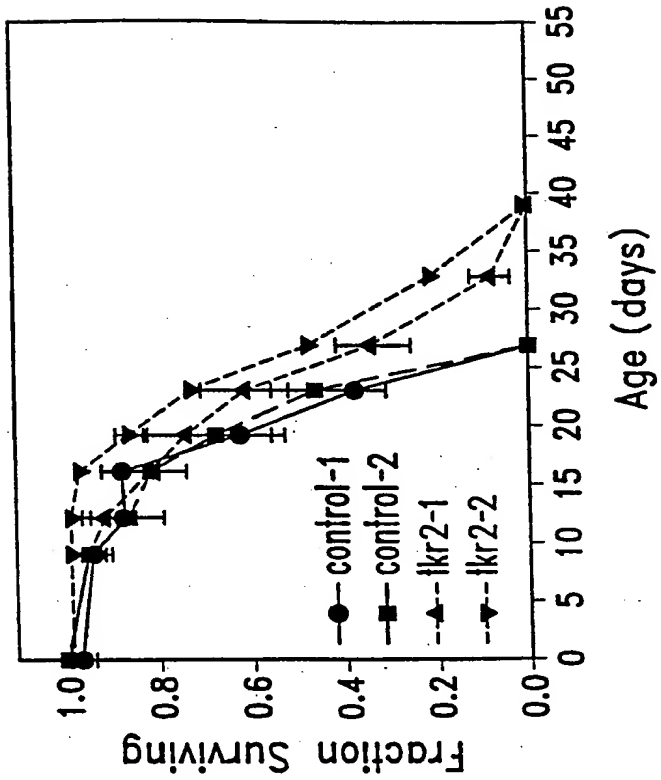

Fig. 9b

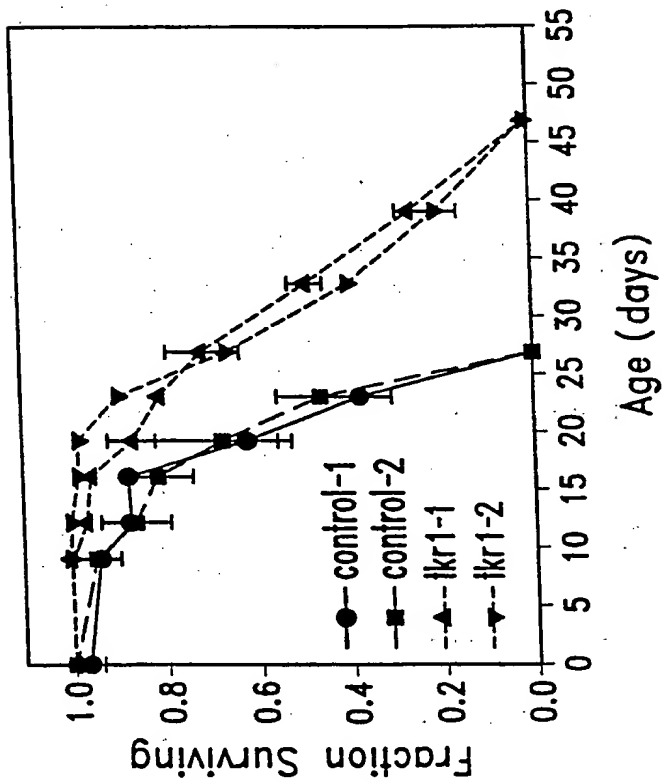

Fig. 9a

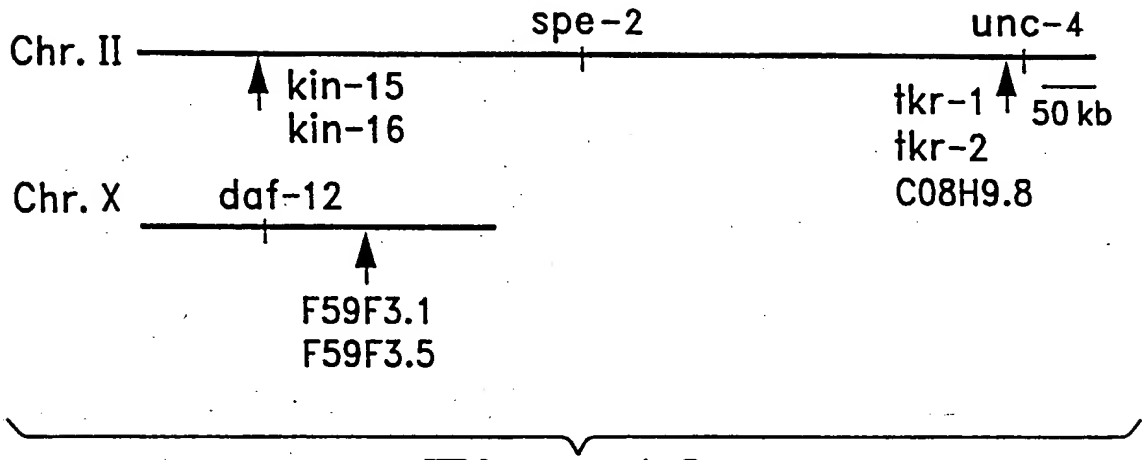

Fig. 10a

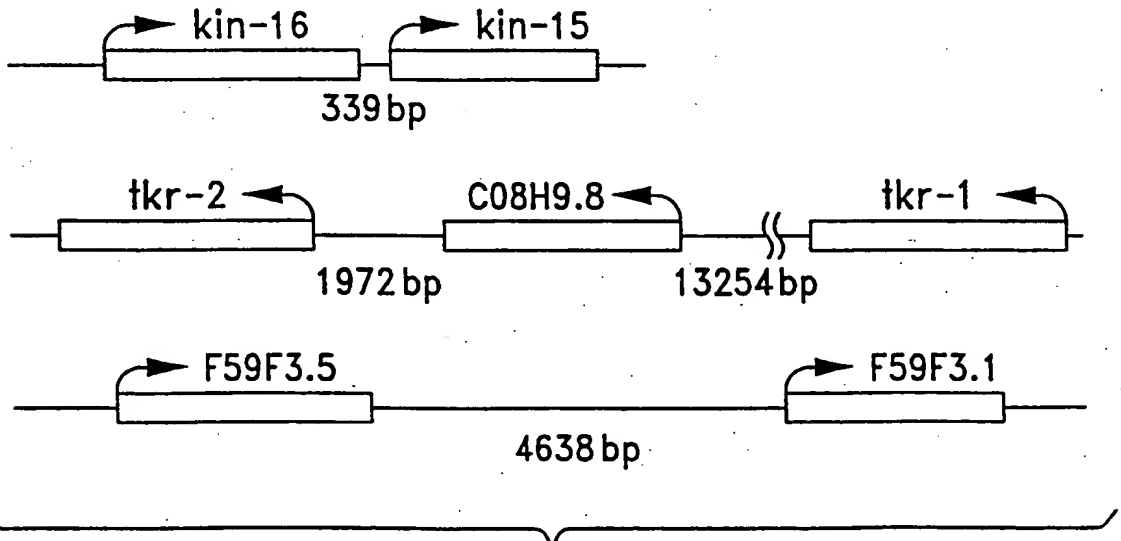

Fig. 10b

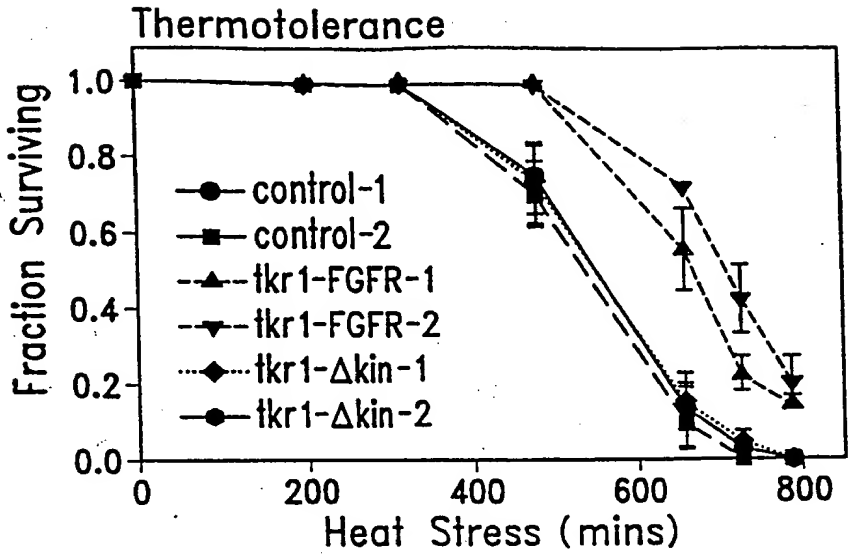

Fig. 11a

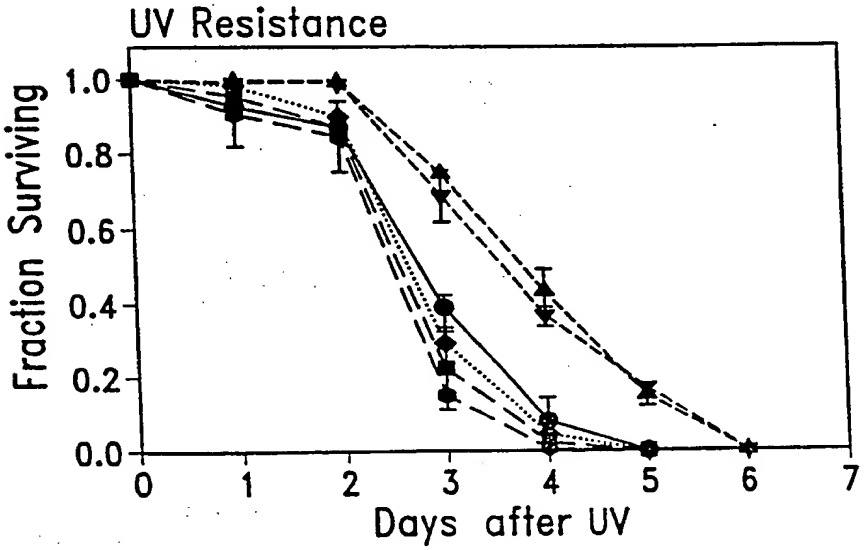

Fig. 11b

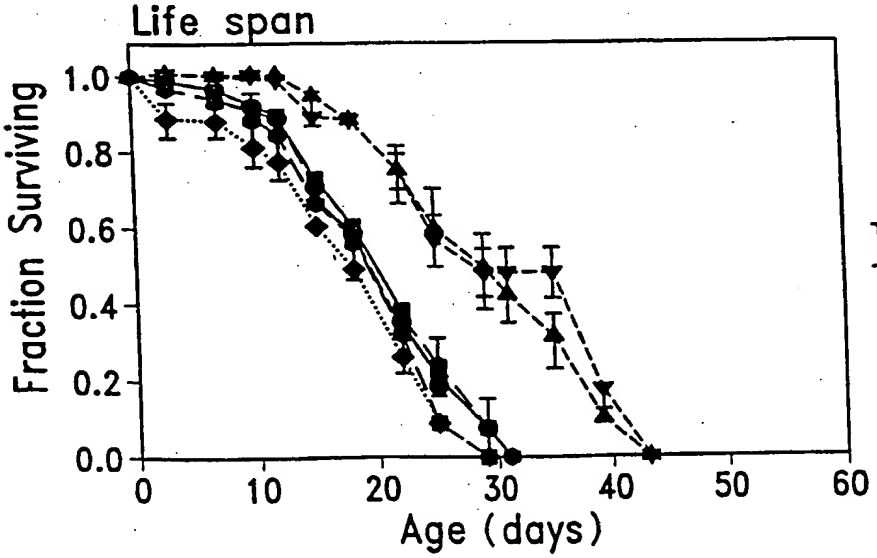

Fig. 11c

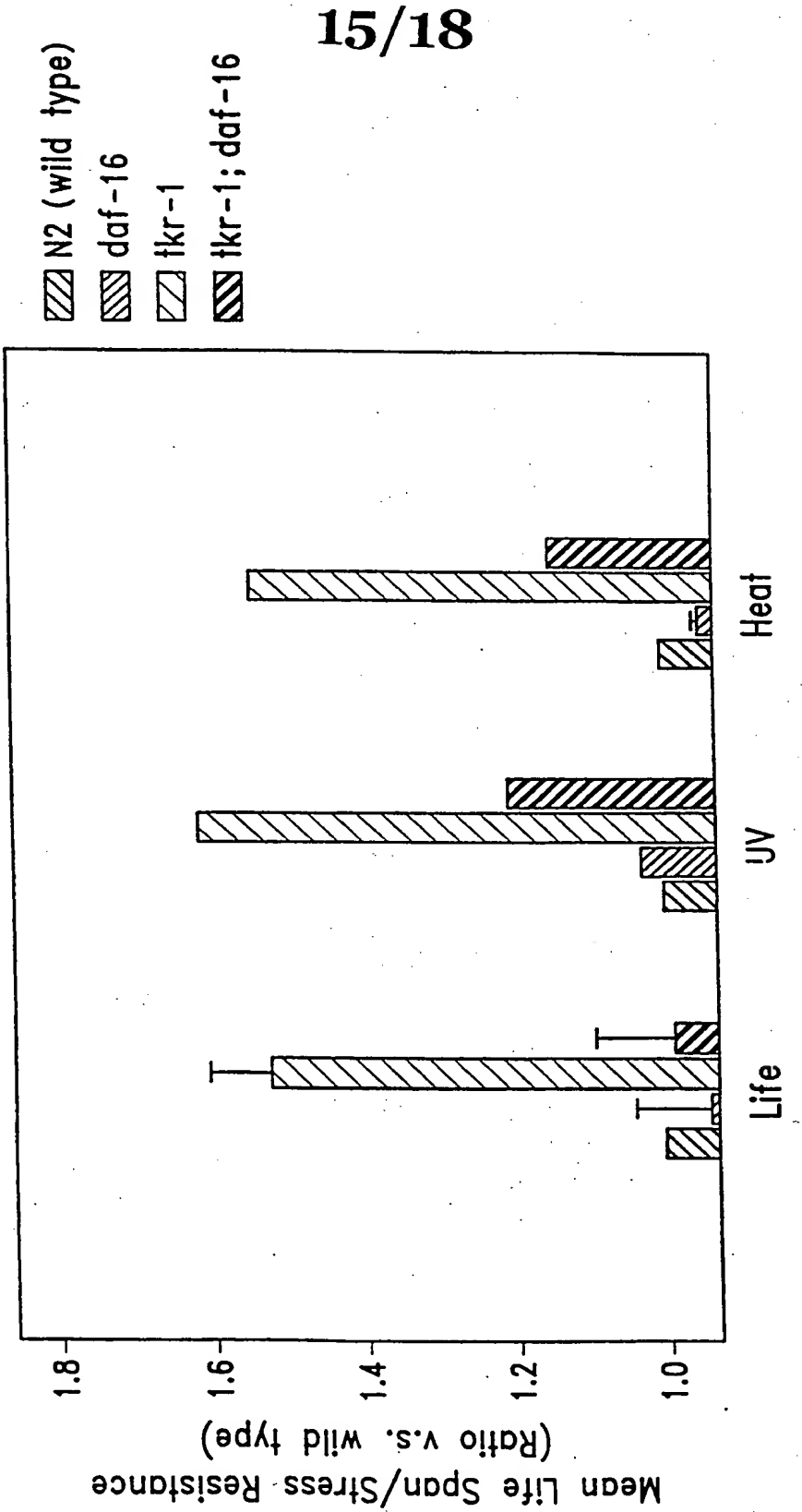

Fig. 12

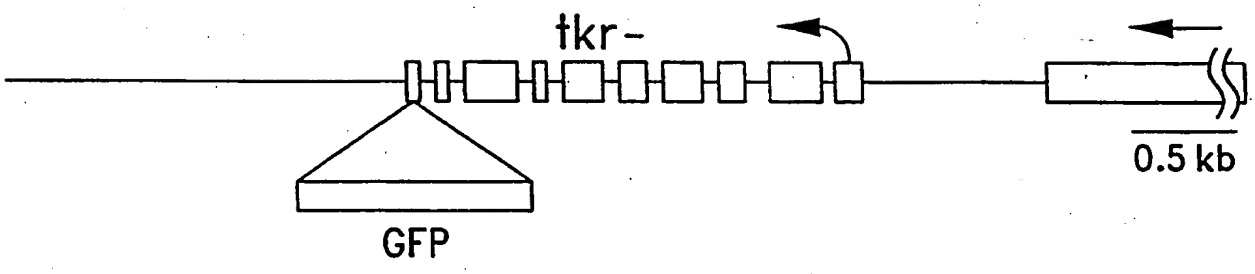

Fig. 13

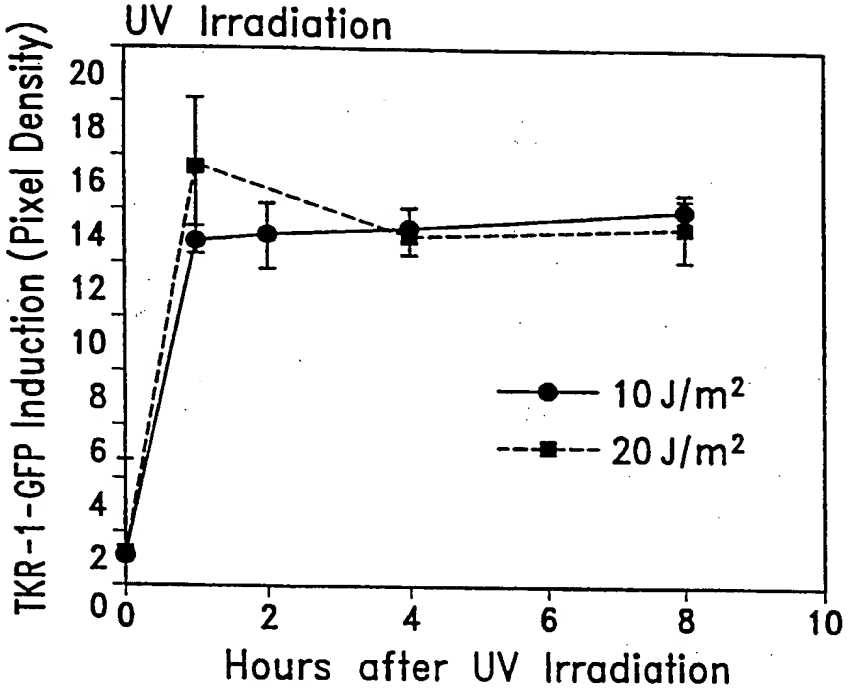

Fig. 14a

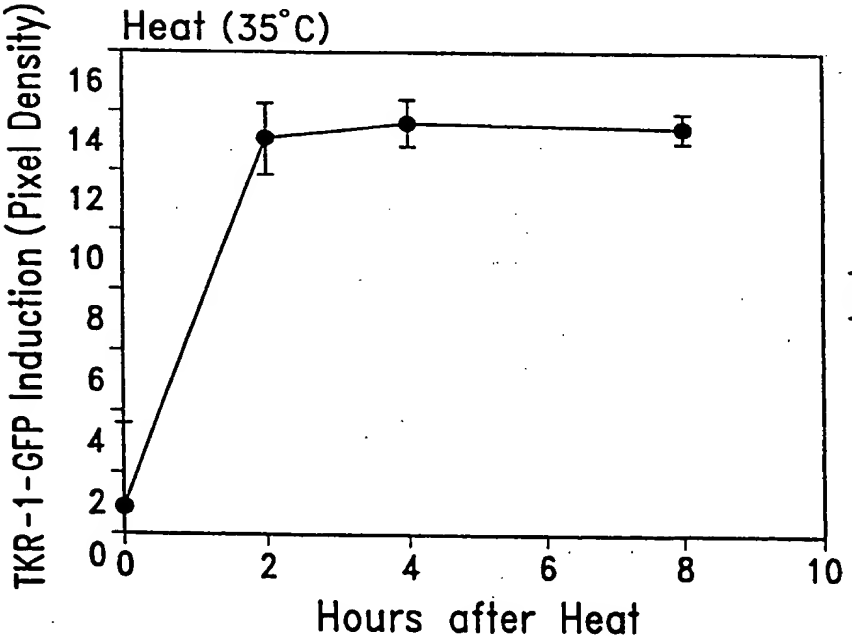

Fig. 14b

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Fig. 15

Arg 1	Leu	Val	Leu	Gly 5	Lys	Pro	Leu	Gly	Glu 10	Gly	Cys	Phe	Gly	Gln 15	Val
Val	Leu	Ala	Glu 20	Ala	Ile	Gly	Leu	Asp 25	Lys	Asp	Lys	Pro	Asn 30	Arg	Val
Thr	Lys	Val 35	Ala	Val	Lys	Met	Leu 40	Lys	Ser	Asp	Ala	Thr 45	Glu	Lys	Asp
Leu	Ser 50	Asp	Leu	Ile	Ser	Glu 55	Met	Glu	Met	Met	Lys 60	Met	Ile	Gly	Lys
His 65	Lys	Asn	Ile	Ile	Asn 70	Leu	Leu	Gly	Ala	Cys 75	Thr	Gln	Asp	Gly	Pro 80
Leu	Tyr	Val	Ile	Val 85	Glu	Tyr	Ala	Ser	Lys 90	Gly	Asn	Leu	Arg	Glu 95	Arg
Leu	Val	Leu	Gly 100	Lys	Pro	Leu	Gly	His 105	Asn	Pro	Glu	Glu	Gln 106	Leu	Ser
Ser	Lys	Asp 110	Leu	Val	Ser	Cys	Ala 115	Tyr	Gln	Val	Ala	Arg 120	Gly	Met	Glu
Tyr	Leu 125	Ala	Ser	Lys	Lys	Cys 130	Ile	His	Arg	Asp	Leu 135	Ala	Ala	Arg	Asn
Val 140	Leu	Val	Thr	Glu	Asp 145	Asn	Val	Met	Lys	Ile 150	Ala	Asp	Phe	Gly	Leu 155
Ala	Arg	Asp	Ile	His 160	His	Ile	Asp	Tyr	Tyr 165	Lys	Lys	Thr	Thr	Asn 170	Gly
Arg	Leu	Pro	Val 175	Lys	Trp	Met	Ala	Pro 180	Glu	Ala	Leu	Phe	Asp 185	Arg	Ile
Tyr	Thr	His 190	Gln	Ser	Asp	Val	Trp 195	Ser	Phe	Gly	Val	Leu 200	Leu	Trp	Glu
Ile	Phe 205	Thr	Leu	Gly	Gly	Ser 210	Pro	Tyr	Pro	Gly	Val 215	Pro	Val	Glu	Glu
Leu 220	Phe	Lys	Leu	Leu	Lys 225	Glu	Gly	His	Arg	Met 230	Asp	Lys	Pro	Ser	Asn 235
Cys	Thr	Asn	Glu	Leu 240	Tyr	Met	Met	Met	Arg 245	Asp	Cys	Trp	His	Ala 250	Val
Pro	Ser	Gln	Arg 255	Pro	Thr	phe	Lys	Gln 260	Leu	Val	Glu	Asp	leu 265	Asp	Arg
Ile	Val	Ala 270	Leu	Thr	Ser	Asn	Gln 275	Glu	Tyr	Leu	Asp	Leu 280	Ser	Met	

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 98/14223

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C12N15/54 C12N9/12 A01K67/033
G01N33/68 C12Q1/48 C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOHNSON T. E. ET AL.: "Genetics of aging and longevity in lower organisms" CELLULAR AGING AND CELL DEATH, vol. 16, 1996, pages 1-17, XP002087514	1-5
Y	see page 2, right-hand column, paragraph 3 - page 4, left-hand column, paragraph 1 see page 10, right-hand column, paragraph 2 - page 12, left-hand column, paragraph 2 see page 12, right-hand column, line 21 - page 13, left-hand column, line 5 ---	8,11-15
Y	WO 93 25694 A (MASSACHUSETTS INST. TECHNOLOGY (US); HORVITZ H.R.; YUAN J.; SHAHAM S.) 23 December 1993 see page 16, line 4 - page 23, line 22 --- -/--	8,11-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 1998	29/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUHON S.A. ET AL.: "Direct isolation of longevity mutants in the nematode Caenorhabditis elegans" DEVELOPMENTAL GENETICS, vol. 18, no. 2, 1996, pages 144-153, XP002087515 see page 152 ---	1-3,7
X	LITHGOW G.J. ET AL.: "Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 92, August 1995, pages 7540-7544, XP002087516 cited in the application see page 7543, right-hand column, paragraph 2-3 see page 7544, left-hand column, paragraph 2-4 ---	1-5
X	JOHNSON T.E. AND SHOOK D.R.: "Identification and mapping of genes determining longevity" BETWEEN ZEUS AND THE SALMON: THE BIODEMOGRAPHY OF LONGEVITY. WATCHER K.W. AND CALEB E.F. EDS. NATIONAL ACADEMY PRESS WASHINGTON D.C.,1997, pages 108-126, XP002087517 see page 109, paragraph 3 - page 110, paragraph 1; tables 7-1 see page 114, paragraph 3 - page 115 see page 118, paragraph 3 - page 119, paragraph 1 ---	1-3
A	JOHNSON T.E.: "Genetic influences on aging" EXPERIMENTAL GERONTOLOGY, vol. 32, no. 1/2, January 1997 - April 1997, pages 11-22, XP002087518 see page 12, paragraph 2 see page 17, paragraph 3 - page 19 ---	1-3
T	MURAKAMI S. AND JOHNSON T.E.: "Life extension and stress resistance in Caenorhabditis elegans modulated by tkr-1 gene" CURRENT BIOLOGY, vol. 8, no. 19, 24 September 1998, pages 1091-1094, XP002087519 ---	

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1

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/14223

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MURAKAMI S. ET AL.: "A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans" GENETICS, vol. 143, no. 3, July 1996, pages 1207-1218, XP002054193 cited in the application ---	
A	MORRIS J.Z. ET AL.: "A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans" NATURE, vol. 382, 8 August 1996, pages 536-539, XP002087520 cited in the application -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 14223

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Remark: in light of the description, Seq.ID:2 cited in claim 9 and Seq.ID:3 cited in claim 10 should be Seq.ID:12 and 13, respectively. The search of the subject matter of claims 9 and 10 was performed accordingly.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/US 98/14223

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9325694 A	23-12-1993	EP 0672151 A	20-09-1995
		JP 8500482 T	23-01-1996
		WO 9325685 A	23-12-1993